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Research Article

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Activation of Endogenous ΔF508 Cystic Fibrosis Transmembrane Conductance Regulator by Phosphodiesterase Inhibition

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Abstract

Many heterologously expressed mutants of the cystic fibrosis transmembrane conductance regulator (CFTR) exhibit residual chloride channel activity that can be stimulated by agonists of the adenylate cyclase/protein kinase A pathway. Because of clinical implications for cystic fibrosis of activating mutants in vivo, we are investigating whether ΔF508, the most common disease-associated CFTR mutation, can be activated in airway epithelial cells. We have found that $^{36}Cl^-$ efflux can be stimulated 19–61% above baseline by β-adrenoreceptor agonists and cGI-phosphodiesterase inhibitors in transformed nasal polyp (CF-T43) cells homozygous for the ΔF508 mutation. The increase in $^{36}Cl^-$ permeability is diminished by protein kinase A inhibitors and is not mediated by an increase in intracellular calcium concentrations. Preincubation of CF-T43 cells with CFTR antisense oligonucleotides prevented an increase in $^{36}Cl^-$ efflux in response to β-agonist and phosphodiesterase inhibitor. Primary cells isolated from CF nasal polyps gave similar results. These data indicate that endogenous levels of ΔF508 protein can be stimulated to increase $^{36}Cl^-$ permeability in airway epithelial cells. (*J. Clin. Invest.* 1996. 98:513–520.)

Key words: cystic fibrosis • epithelium • ion channels • cyclic AMP • protein kinases

Introduction

Cystic fibrosis is a recessive, inherited disorder caused by mutations in the gene encoding a cyclic adenosine monophosphate (cAMP)-dependent, phosphorylation-regulated chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR)[1] (1–4). The disease is characterized by abnormal fluid and electrolyte transport across the epithelia of numerous tissues (5, 6), consistent with the idea that the primary defect in CF patients is the reduction or absence of epithelial chloride channels due to mutations in the CFTR gene (reviewed in reference 7). It is predicted that restoring electrolyte and fluid transport to more normal levels will ameliorate CF symptoms to some degree. Consequently, numerous therapeutic approaches for CF have been suggested in which modulating electrolyte transport, either pharmacologically, or by gene replacement therapy, is the target. Some of the strategies suggested or under investigation for improving electrolyte transport in CF include gene transfer of a normal CFTR cDNA to CF cells (8–11) or pharmacological manipulation of ion channels other than CFTR, such as amiloride-sensitive sodium channels (12) and purine-activated chloride channels (13). A third approach is suggested by the observation that many disease-related CFTR mutants possess some residual channel activity that can be induced by stimulating the cAMP/protein kinase A (PKA) pathway (14–18). This raises the possibility that inducing chloride transport in vivo through mutant CFTRs by pharmacological manipulation of the cAMP–PKA pathway may be a therapeutic alternative for CF (14–18). This is an important approach to pursue, as the majority of the CF population (~95%) carry at least one mutation shown to have residual chloride channel activity.

Pharmacologically targeting activation of CFTR requires identifying and understanding the components of the pathways to be manipulated. We have recently demonstrated that cGMP-inhibited phosphodiesterases (cGI-PDE) are involved in the regulatory pathway of CFTR in Calu-3 and 16HBE human airway epithelial cells (19), apparently by regulating cAMP levels and PKA activity in specific cellular compartments. Dephosphorylation is also involved in the regulation of CFTR, and at least some of the phosphatases controlling the process appear to be localized to the plasma membrane (14). For instance, protein phosphatase 2A (PP2A) affects CFTR activity to a greater extent than protein phosphatases 1 or 2B (PP1, PP2B) in National Institutes of Health 3T3 fibroblasts stably expressing recombinant CFTR (20), whereas CFTR expressed in cardiac myocytes appears to be controlled by PP2A and PP2C (21). These examples illustrate that CFTR regulation is dependent on specific isoforms of several proteins associated with the cAMP–PKA pathway.

The ability to activate disease-causing CFTR mutants has been demonstrated in a variety of cell types by manipulating enzyme activities in the cAMP–PKA pathway with specific pharmacological agents. The CFTR mutants R117H and G551D expressed in chinese hamster ovary cells were activated by the phosphatase inhibitor (-)-*p*-bromotetramisole in the absence of cAMP elevating agents (14). (-)-*p*-Bromotetramisole slowed the rundown of these mutants in excised patches, implying not only a functional relationship between CFTR and phosphatases, but also close proximity of the proteins within the plasma membrane. Sheppard et al. (15, 16) were able to activate a number of mutant CFTRs associated

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1. *Abbreviations used in this paper:* BAPTA-AM, [1,2-*bis*-(*o*-Aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid tetra-(acetoxymethyl)-ester]; CFNP, cystic fibrosis nasal polyp; CFTR, cystic fibrosis transmembrane conductance regulator; cGI, cyclic GMP-inhibited; H-8, *N*-[2-methylamino)ethyl]-5-isoquinoline sulfonamide; HBR, Hepes-buffered Ringer's; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; PKA, protein kinase A; PP, protein phosphatase.

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with pancreatic sufficiency, and demonstrated that these milder mutations have residual activity that can be observed by stimulating the cAMP–PKA pathway or exposing the channels to PKA directly. In *Xenopus* oocytes (17), CFTR mutants were activated with a combination of the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). This combination of drugs activated CFTRs carrying the disease-causing mutations G551S, G551D, and ΔF508, as well as F508C and wild type. In these experiments, activation of ΔF508 and G551D required extremely high concentrations of IBMX and the oocytes were cultured at 19°C, reducing the processing defect associated with the ΔF508 mutation in cells grown at 37°C (22, 23). However, activation of the ΔF508 mutation was reported in Vero (18, 24) and C127 cells (25) cultured at 37°C.

These examples demonstrate that mutant CFTR activation is possible and may be a feasible approach to CF therapy. It is important to note that each of these studies used heterologous expression systems to generate CFTR and its mutants and, in some cases, lower incubation temperatures to augment processing of ΔF508. Activation of endogenously expressed ΔF508 CFTR through manipulation of the cAMP–PKA pathway has not been previously reported. Here we demonstrate that a combination of β-adrenoreceptor agonist and cGI-PDE inhibitor can stimulate cAMP-mediated chloride transport in the transformed CF nasal polyp cell line CF-T43, homozygous for the ΔF508 mutation. Depletion of CFTR by culturing cells in the presence of CFTR-specific antisense oligonucleotides reduced to baseline any chloride transport in response to β-agonist and PDE inhibition. Virtually identical results were obtained when primary CF nasal polyp cells from a patient homozygous for the ΔF508 mutation were exposed to these conditions as well.

Methods

Cell culture

CF-T43 cells (26) were generously provided by Dr. James Yankaskas at the University of North Carolina (Chapel Hill, NC) and grown in keratinocyte basal medium (KBM; Clonetics Corp., San Diego, CA) containing epidermal growth factor (0.1 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), calcium (0.15 mM), bovine pituitary extract, and amphotericin-B (50 ng/ml) (KGM BulletKit, Clonetics Corp.). Cells were grown with 5% CO_2 at 37°C. CF nasal polyp (CFNP) cells were obtained from polypectomies in which epithelial cells were isolated by dissociating cells in a solution of DNase and collagenase as described elsewhere (27). Cells were plated and fibroblasts allowed to attach for 60 min, at which time nonadherent cells (predominantly epithelial) were transferred to a collagen-coated plate and allowed to adhere overnight. For efflux experiments, cells were seeded at 0.5×10^6 cells per well of a six-well culture dish (Falcon Labware, Cockeysville, MD) coated with human placental collagen. CFNP cells were grown in bronchial/tracheal epithelial cell growth medium (BEGM; Clonetics Corp.) containing bovine pituitary extract, insulin (5 mg/ml), hydrocortisone (0.5 mg/ml), retinoic acid (1 μg/ml), transferrin (10 mg/ml), T3 (6.5 μg/ml), epinephrine (0.5 mg/ml), and epidermal growth factor (0.5 ng/ml) (BEGM BulletKit) that was supplemented with tobramycin (20 μg/ml). Cells were grown with 5% CO_2 at 37°C.

Chloride efflux assay

The chloride efflux assay used in these studies is a modification of that described by Venglarik et al. (28), in which ^{36}Cl is substituted for ^{125}I. Cells were seeded at 10^5 cells/well of six-well culture dishes (35 mm; Falcon Labware) and grown to confluency. Cells were incubated for 2 h at 37°C in 1 ml Hepes-buffered Ringers solution (HBR) consisting of 10 mM Hepes, 138 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM glucose containing 5 μCi $Na^{36}Cl$ (Amersham Corp., Arlington Heights, IL). After incubation, cells are washed four times with 1 ml HBR to remove extracellular ^{36}Cl. Values of isotopic efflux are recorded after the fourth wash. Efflux was measured at 37°C in 30-s intervals, where HBR was removed from the cells, transferred to a scintillation vial, and replaced with fresh HBR. Agonists were added at the indicated time point. After the final efflux interval, cells were lysed with 1% Triton X-100 in HBR for 20 min at 37°C. Scintillation fluid was added and vials were counted in a liquid scintillation counter (LS 5801; Beckman Instruments, Inc., Fullerton, CA). Efflux was calculated as the percentage of ^{36}Cl lost from the cells per 30-s interval from ^{36}Cl remaining in the cells and plotted as a function of time. Effects of drugs on efflux were calculated as the area under the curve corresponding to the presence of drugs and dividing by the average area for control. The ratios were evaluated statistically by analysis of variance and the means were compared using ANOVA and Bonferroni/Dunn test.

PKA inhibition. Experiments using *N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide (H-8) (Sigma Chemical Co., St. Louis, MO) and adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-cAMP) were performed by preincubating cells for 30 min before the efflux assay at 37°C with 100 μM of the compound. The inhibitors were also present through the duration of the efflux assay.

Calcium chelation. To determine the role of intracellular calcium in chloride transport, efflux experiments were performed in the presence of [1,2-*bis*-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra-(acetoxymethyl)-ester] (BAPTA-AM) (Calbiochem Corp., La Jolla, CA). Cells were preincubated for 30 min at 37°C in the presence of BAPTA-AM before the start of efflux. All other conditions remained as previously described.

CFTR sense/antisense oligonucleotide studies. Oligonucleotides were synthesized on a DNA synthesizer (Oligo 1000; Beckman Instruments, Inc.). Sequences were chosen from the first 18 and second 18 base pairs of exon 1 of CFTR (2). Sense oligonucleotide were 5′-ATG CAG AGG TCG CCT CTG-3′ and 5′-GAA AAG GCC AGC GTT GTC-3′. Antisense oligonucleotides used were 5′-CAG AGG CGA CCT CTG CAT-3′ and 5′-GAC AAC GCT GGC CTT TTC-3′. CF-T43 cells and CFNP cells were cultured for 48 h in the presence of 12 μM oligonucleotides before efflux. Fresh oligonucleotide was added every 12 h. This method has previously been shown to greatly reduce CFTR expression in a variety of cell types expressing CFTR (29–33).

cAMP assays

Cells were grown in 24-well culture dishes (Corning Glassworks, Corning, NY) to confluency. PDE inhibitors were added to a final concentration of 100 μM and isoproterenol to 10 μM and cells incubated for 15 min at 37°C. Media was removed and 0.5 ml HCl (0.1 N) was added to the wells and left overnight at room temperature to lyse cells. cAMP assays were carried out essentially as described by Davis et al. (34). Increases in cAMP are calculated as the ratio of cAMP from stimulated cells, in femtomoles per milliliter, to baseline cAMP.

Fluorescent microscopy

CF-T43 cells were grown to subconfluence on round glass coverslips under tissue culture conditions. Cells were loaded with 10–20 μM Fura-2–acetoxymethyl ester (Fura-2-AM) in HBR at room temperature for 1 h. Loaded coverslips were washed three times with HBR to remove extracellular dye. Coverslips were then placed in a modified Sykes-Moore chamber (Bellco Biotechnology, Vineland, NJ) and perfused by gravity at a rate of 6 ml/min with HBR. Experiments were performed at 37°C and viewed with an upright epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Fluorescence was excited alternately at 340 and 380 nm. The emission wavelength was 510 nm. Image-1/F1 software (Universal Imaging Corp., Media, PA) was

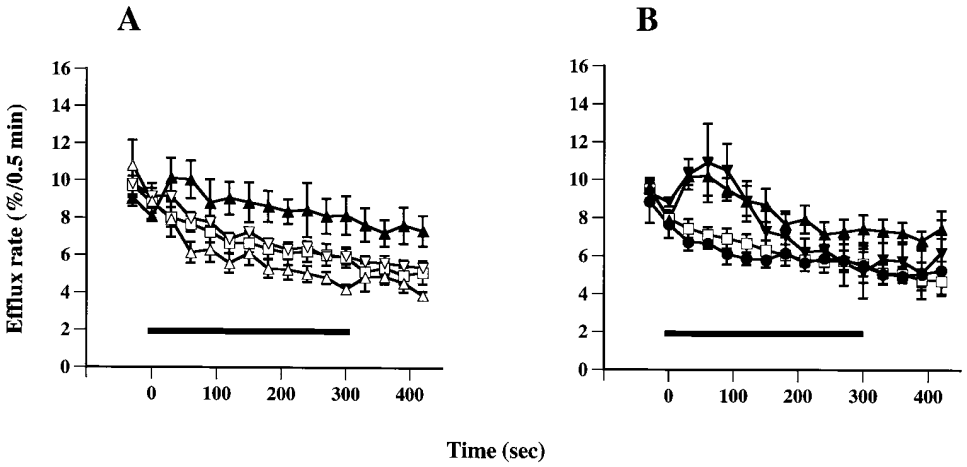

Figure 1. PDE inhibitor–β agonist synergism and PDE inhibitor class specificity requirements for the activation of chloride transport in CF-T43 cells. ^{36}Cl efflux rate is plotted as a function of time, and the addition of stimulatory compounds is indicated by the solid bar. Error bars represent SEM. Control experiments represent efflux assays performed in the absence of any agonists ($n = 11$). (*A*) Synergistic effects of β agonist and PDE inhibitor are evident as neither isoproterenol ($n = 3$) nor milrinone ($n = 3$) alone increased efflux significantly, while the same concentrations added simultaneously ($n = 11$) gave a significant increase. (*B*) Syner gism is specific for cGI-PDEs, as either milrinone ($n = 3$) or amrinone ($n = 3$), both cGI-PDE inhibitors, increase efflux when in combination with isoproterenol, while IBMX ($n = 3$) with isoproterenol has no significant effect. PDE inhibitors were used at 100 μM; isoproterenol at 10 μM. Control (□); isoproterenol (▼); milrinone (△); isoproterenol+milrinone (▲); isoproterenol+amrinone (▼); and isoproterenol+IBMX (●).

used to analyze the images. Upon attaining a steady state of fluorescence from a group of seven to nine defined regions, each region consisting of a separate cell, the cells were perfused with HBR containing the agonists 10 μm albuterol and 100 μm milrinone, or 1 μm ionomycin for 10 min, and then reperfused with only HBR thereafter. In experiments performed in the presence of the calcium chelator BAPTA-AM (5 μM), cells were preincubated for 30 min at room temperature with BAPTA-AM with all other conditions remaining as described. Background fluorescence was subtracted from all data.

Short circuit measurements

CF-T43 cells were seeded at a density of 8×10^5 cells/cm^2 on Millicell-CM permeable supports from Millipore Corp. (Bedford, MA). Cells were grown in culture as described above and used between 10 and 14 d after seeding. Voltage clamping was carried out using a DVC 1000 voltage/current clamp from WPI (Sarasota, FL). Cells were mounted in a modified Ussing chamber (WPI) with KRB consisting of 25 mM $NaHCO_3$, 115 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM glucose, pH 7.4. All experiments were carried out at 37°C and KRB was continuously bubbled with 95% O_2/5% CO_2 by air lift circulators. Transepithelial voltage (V_t) was recorded through agar bridges (4% agar in KRB) connected to balanced calomel electrodes. V_t was clamped to 0 and short circuit current (I_{sc}) was recorded through Ag–AgCl electrodes connected to the chamber via agar bridges. Transepithelial resistance (R_t) was calculated by measuring the change in I_{sc} (ΔI_{sc}) in response to a +2 mV clamp of V_t (3-s pulse every 30 or 40 s). Data were collected on a MacLab/4e from Advanced Instruments Inc. (Milford, MA).

Results

The activation of ΔF508 in *Xenopus* oocytes requires a combination of adenylate cyclase agonist and high concentrations of phosphodiesterase inhibitor. We investigated the possibility that a similar approach could be used to activate ΔF508 in airway epithelial cells. Previously (19), we reported that inhibitors of cGI-PDEs have the greatest impact on CFTR activity in airway cells, so the ability of these compounds to activate ΔF508 was tested using the cell line CF-T43, derived from a CF nasal polyp, as a model system. As Fig. 1 *A* shows, the cGI-PDE inhibitor milrinone has no effect on chloride efflux from CF-T43 cells when applied alone, but as in the *Xenopus* oocytes, when combined with an adenylate cyclase agonist such as isoproterenol, a significant increase in efflux is observed. This increase is not due to adenylate cyclase activity alone, as isoproterenol had no effect without the addition of PDE inhibitor.

As was found in airway cells expressing wild-type CFTR, activation by PDE inhibitors occurred through inhibition of cGI-PDEs, as shown by the similarity of responses to amrinone and milrinone, both inhibitors specific for cGI-PDEs, and the lack of effect by IBMX, a relatively nonspecific PDE inhibitor (Fig. 1 *B*). Also, the class IV–specific inhibitors rolipram and Ro20-1724 had no significant effect on efflux (data not shown). These results are consistent with previous reports in which IBMX failed to activate chloride currents across CF airway epithelial cells (35).

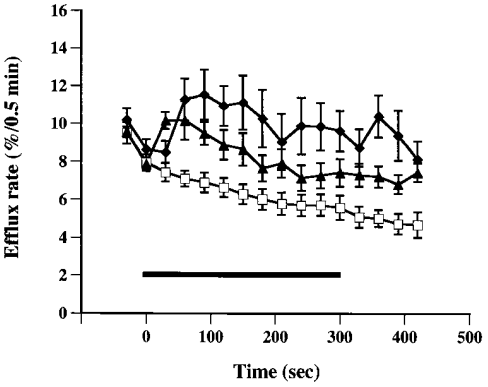

Figure 2. Effects of two different β agonists on chloride efflux stimulation in CF-T43 cells. The solid bar indicates the presence of stimulatory compounds in the assay. Control experiments ($n = 11$) are performed in the absence of agonists. Either isoproterenol ($n = 11$) or albuterol ($n = 9$) (10 μM) in combination with milrinone (100 μM) generate effluxes significantly higher than control. Error bars represent SEM. Control (□); isoproterenol+milrinone (▲); and albuterol+milrinone (◆).

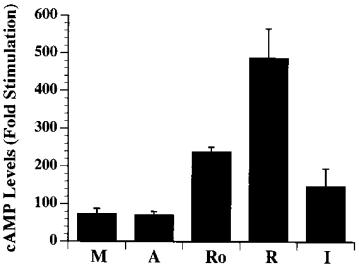

Figure 3. Stimulation of cAMP in CF-T43 cells. Cells were exposed to 10 μM isoproterenol and 100 μM PDE inhibitor (*M*, milrinone; *A*, amrinone; *Ro*, Ro20-1724; *R*, rolipram; and *I*, IBMX) for 15 min, after which cAMP levels were measured. The ratio of cAMP after stimulation to cAMP without stimulation is represented as "fold stimulation." $n = 3$ for each condition; error bars represent SEM.

The synergistic action between isoproterenol and the cGI-PDE inhibitors is not idiosyncratic to isoproterenol, an agonist for both β_1 and β_2 adrenoreceptors. Other β-adrenoreceptor agonists such as albuterol, which is specific for β_2 adrenoreceptors, also stimulate efflux (Fig. 2). In these experiments, chloride efflux appeared to be of larger magnitude when albuterol was used as an agonist, possibly indicating the involvement of β_2 receptors in this process. Alternatively, this effect may be due to receptor desensitization known to occur with isoproterenol (36).

The requirement for both PDE inhibition and adenylate cyclase activation indicates a cAMP-dependent process. cAMP levels were measured in response to the various PDE inhibitors (100 μM) in combination with isoproterenol (10 μM), which are the conditions used for efflux, to determine the relationship between cAMP and chloride efflux. We found that the type IV PDE inhibitors rolipram and Ro20-1724, and the nonspecific inhibitor IBMX raised cAMP 487-, 237-, and 146-fold, respectively, above control values (Fig. 3). Surprisingly, we also found that amrinone and milrinone were not nearly as effective at raising cAMP, producing increases of 70- and 72-fold, respectively.

In addition to CFTR, another major chloride channel of epithelial cells is calcium dependent (37). To determine if activation of calcium-dependent chloride channels was contributing to the observed effects, intracellular calcium activity was measured by fluorescence microscopy with the calcium-sensitive dye FURA-2. CF-T43 cells loaded with FURA-2 (Fig. 4) showed no increase in intracellular calcium levels in response to 10 μM albuterol and 100 μM milrinone, which were sufficient to increase chloride efflux. In contrast, the calcium ionophore, ionomycin (1 μM), caused a significant increase in calcium concentration (Fig. 4). The ionomycin-induced increase in intracellular calcium was completely inhibited by preincubation with BAPTA-AM, the membrane-permeant form of the calcium chelator, BAPTA. Similarly, BAPTA-AM (5 μM) had no effect on efflux stimulated by isoproterenol and milrinone, but eliminated the ionomycin response (Fig. 5). Chloride efflux in the presence of BAPTA-AM (5 μM) was measured in response to either ionomycin (1 μM) or isoproterenol (10 μM) and milrinone (100 μM). As Fig. 5 shows, cells exposed to BAPTA-AM show a significant increase in chloride efflux when stimulated by isoproterenol and milrinone. In contrast, the calcium chelator prevented increases in chloride efflux stimulated by ionomycin (1 μM). These experiments show that chloride efflux stimulated by either albuterol or isoproterenol in combination with milrinone is not due to the activation of calcium-dependent chloride channels in response to elevation of intracellular calcium.

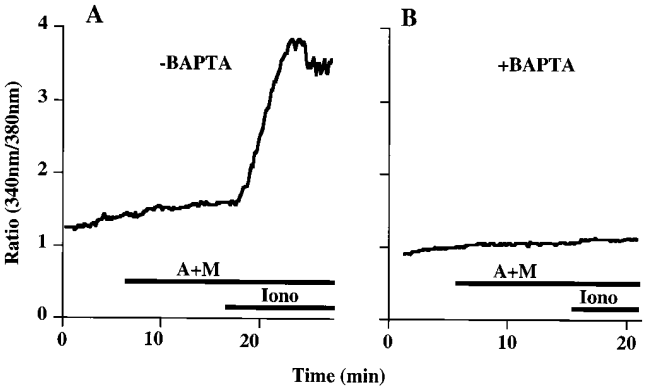

Figure 4. Intracellular calcium activity in CF-T43 cells. Fura-2 fluorescent microscopy was performed as described in Methods. Intracellular calcium measured before and during exposure to 10 μM albuterol plus 100 μM milrinone (*A*+*M*) followed by 1 μM ionomycin (*Iono*) (*A*) with or (*B*) without preincubation in 5 μM BAPTA-AM. Solid bars indicate the presence of A+M or Iono as indicated.

CFTR channel activity is phosphorylation-regulated by PKA. The dependence of efflux on PKA was investigated by treating cells with the kinase inhibitor H-8 (100 μM) and the PKA-specific inhibitor Rp-cAMP (100 μM) and measuring their effects on chloride efflux (Fig. 6). H-8 appears to increase the basal efflux rate in CF-T43 cells, but the typical efflux increase in response to albuterol (10 μM) and milrinone (100 μM) is not observed in the presence of H-8 (Fig. 6 *A*), indicating a kinase-mediated pathway. H-8 inhibits protein kinases by blocking the ATP-binding site, but it is not completely specific for PKA. In these cells, the cAMP analog Rp-cAMP, which binds to the regulatory subunits of PKA, can be used to show the specific dependence of efflux on PKA. As with H-8, Rp-cAMP prevented increased chloride efflux stimulated by isoproterenol and milrinone (Fig. 6 *B*). The combination of these inhibitors indicates that the increase in efflux generated by β

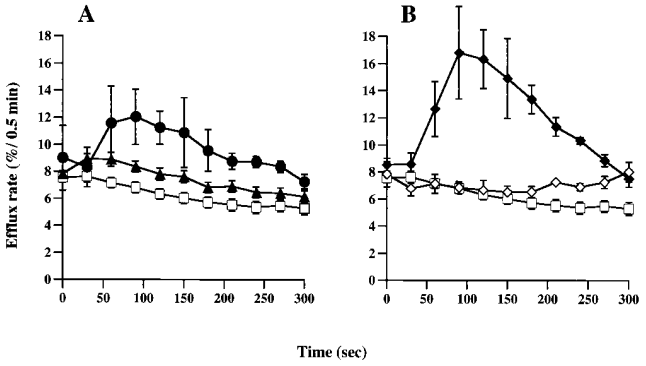

Figure 5. Effect of calcium chelation on ^{36}Cl efflux in CF-T43 cells. Time = 0 corresponds to the addition of stimulatory compounds in the assay. Control experiments ($n = 17$) represent efflux assays performed in the absence of agonists. (*A*) ^{36}Cl efflux stimulation in response to 10 μM isoproterenol plus 100 μM milrinone ($n = 26$) is unaffected by preincubation with BAPTA-AM (30 min, 5 μM, $n = 3$). (*B*) Efflux response to 1 μM ionomycin ($n = 3$) is eliminated by preincubation with BAPTA-AM (30 min, 5 μM, $n = 3$). Error bars represent SEM. Control (□); isoproterenol+milrinone (▲); isoproterenol+milrinone+BAPTA (●); ionomycin (◆); and ionomycin+BAPTA (◇).

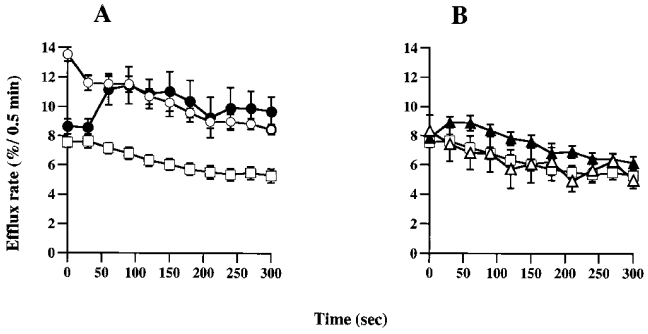

Figure 6. Effect of PKA inhibitors on ^{36}Cl efflux in CF-T43 cells. Time = 0 corresponds to the addition of stimulatory compounds and/or kinase inhibitors in the assay. Control experiments are performed in the absence of agonists. ^{36}Cl efflux stimulation in response to (*A*) isoproterenol (10 μM) plus milrinone (100 μM), in the presence ($n = 3$) or absence ($n = 26$) of Rp-cAMP or (*B*) albuterol (10 μM) plus milrinone (100 μM) in the presence ($n = 5$) or absence ($n = 10$) of H-8 (100 μM). Error bars represent SEM. Control (□); isoproterenol+milrinone (▲); isoproterenol+milrinone+Rp-cAMP (△); albuterol+milrinone (●); and albuterol+milrinone+H-8 (○).

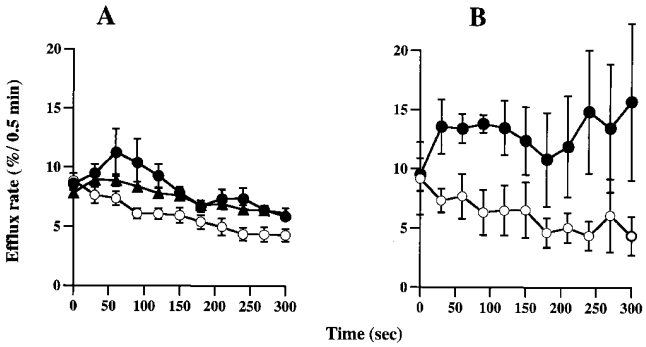

Figure 7. Antisense oligonucleotides to CFTR reduce ^{36}Cl efflux in CF-T43 cells and ΔF508 homozygous CF nasal polyp epithelial cells. Control cells were not exposed to oligonucleotides. Time = 0 corresponds to the addition of 10 μM isoproterenol and 100 μM milrinone in the assay for all experiments. Experiments were conducted as described in Methods for both (*A*) CF-T43 cells ($n = 26$ for control, $n = 7$ each for sense and antisense) and (*B*) CF nasal polyp cells ($n = 3$ for each sense and antisense). Error bars represent SEM. Control (▲); antisense (○); and sense (●).

agonist and PDE inhibitor is not only kinase dependent, but that the kinase involved is PKA.

The observed increases in efflux are dependent on cAMP and PKA, consistent with CFTR activation. However, this would require substantial activation of endogenous levels of the ΔF508 CFTR mutant, a phenomenon that has not been reported previously. To confirm the involvement of CFTR, CF-T43 cells were cultured in the presence of sense or antisense oligonucleotides that correspond to CFTR message (33). Chloride efflux was then measured in response to isoproterenol and milrinone. CF-T43 cells grown in the presence of antisense oligonucleotides showed no response to the combination of milrinone and isoproterenol, (mean efflux rate = 5.94±0.23%/30 s). In contrast, untreated cells, or cells cultured in the presence of sense oligonucleotides, retained cAMP-mediated chloride transport (mean rate = 7.47±0.13 and 8.24±0.34%/30 s, respectively, Fig. 7 *A*). These experiments show that chloride fluxes stimulated by β agonist and the cGI-PDE inhibitor milrinone are mediated by CFTR.

Although not manipulated to alter CFTR expression, the CF-T43 cells are a transformed line and may not respond in the same way as would epithelial cells in vivo. To determine if these results reflect properties of native epithelia, stimulation of chloride transport was attempted in primary CFNP epithelial cells obtained from a CF patient homozygous for the ΔF508 mutation. To identify the source of chloride efflux from these cells, cells were cultured for 48 h in the presence of sense or antisense oligonucleotides corresponding to CFTR message, as was done with the CF-T43 cells. As seen with the CF-T43 cells, isoproterenol (10 μM) and milrinone (100 μM) were able to stimulate chloride permeability in cells treated with sense oligonucleotides (Fig. 7 *B*). However, cells exposed to

Table I. Summary of Efflux Rates

Treatment	*n*	Mean efflux rate	Percent change	*P*
Control	17	6.26±0.14		
Isoproterenol	9	6.63±0.21	6	0.2252
Milrinone	4	5.95±0.38	−5	0.4537
Isoproterenol+milrinone	26	7.47±0.13	19	< 0.0001*
Isoproterenol+amrinone	3	7.90±0.46	26	0.0004*
Isoproterenol+IBMX	4	6.15±0.16	−2	0.7952
Albuterol+milrinone	10	10.08±0.34	61	< 0.0001*
Isoproterenol+milrinone+BAPTA	3	9.60±0.50	53	< 0.0001*
Ionomycin	3	11.76±0.70	88	< 0.0001*
Ionomycin+BAPTA	3	7.07±0.17	13	0.0803
Isoproterenol+milrinone+Rp-cAMPS	3	6.29±0.31	0	0.9522
Isoproterenol+milrinone+CFTR antisense	7	5.94±0.23	−5	0.3353
Isoproterenol+milrinone+CFTR sense	7	8.24±0.34	32	< 0.0001*

The average efflux rates are given in %/30 s±SEM for the time period during which cells are exposed to the various compounds (treatment). Mean rates represent 12 time points over 6 min for each experiment, n = the number of experiments. Percent change corresponds to the percent increase or decrease relative to untreated (control) cells. Statistical significance was determined by ANOVA and comparison of means using the Bonferroni/Dunn test. Significance was set at $\alpha = 0.05$, which corresponds to a P value of 0.006 due to Bonferroni correction for multiple comparisons. *Mean rates significantly different from control.

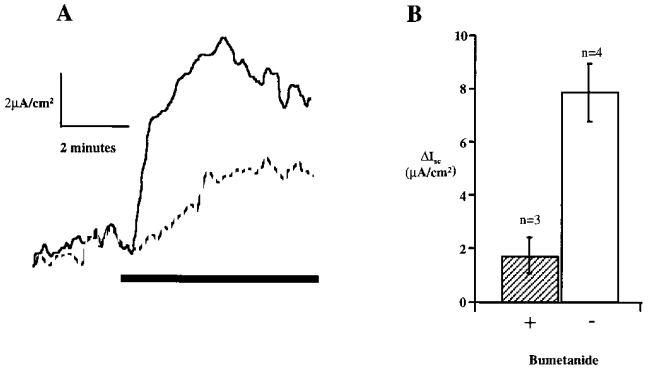

Figure 8. Short circuit currents generated by isoproterenol and milrinone. Monolayers of CF-T43 cells were grown on permeable supports and exposed to 10 μM isoproterenol and 100 μM milrinone with or without pretreatment by 100 mM bumetanide. (*A*) Representative response to isoproterenol and milrinone (*solid bar*) of cells with (*dashed line*) or without (*solid line*) pretreatment with bumetanide. (*B*) Summary of changes in short circuit currents (ΔI_{sc}) stimulated by isoproterenol and milrinone. The hatched bar shows the response when cells are pretreated with bumetanide and the open bar corresponds to the change in current in the absence of bumetanide. Error bars indicate standard error of the mean. Resistances were 14.9±1.49 and 20.05±4.39 Ω/cm² for the bumetanide-treated and -untreated cells, respectively.

antisense CFTR oligonucleotides lost the cAMP-mediated chloride efflux response, indicating that the combination of β agonist and cGI-PDE inhibitor can activate ΔF508 CFTR at endogenous levels in primary CF cells.

The mean efflux rates corresponding to the 5 min of stimulation for the various conditions are shown in Table I. Comparison of the rates between control (untreated cells) and those exposed to various compounds shows that stimulation of CF-T43 cells by β agonists and cGI-PDE inhibitors increases the average efflux rate 19–61% above control values. Neither isoproterenol (10 μM) nor milrinone (100 μM) used alone increase chloride permeability (6%, $P = 0.225$, and −5%, $P = 0.454$, respectively) above control values, demonstrating the synergism required for ΔF508 CFTR activation.

Another method to monitor CFTR activity in a population of cells is by short circuit current (I_{sc}) measurements that, unlike the efflux assay, show vectorial chloride transport as should be found in epithelia in vivo. CF-T43 monolayers were grown on permeable supports and I_{sc} measured in response to 10 μM isoproterenol and 100 μM milrinone, as shown in Fig. 8. The combination of isoproterenol and milrinone increased I_{sc} by 7.85±1.08 μA/cm², whereas monolayers treated with bumetanide (100 μM), which blocks transepithelial chloride secretion by inhibiting chloride entry across the basolateral membrane, showed an increase of only 1.71±0.67 μA/cm². The results indicate that the β agonists combined with inhibitors of cGI-PDEs can increase chloride permeability as well as electrogenic chloride transport across monolayers of cells expressing the ΔF508 mutation.

Discussion

Identification and cloning of the CFTR gene brought with it the possibility that correction of the chloride transport defect in CF can be achieved. With correction of the chloride transport defect, it is predicted that CF-related symptoms will be alleviated and that the progression of disease will be at least delayed. Initial efforts to correct ion transport have focused mainly on gene transfer therapy. Although the therapeutic potential of effective gene transfer therapy is obvious, the problems of low transfer efficiency (38) and the relatively short duration of transferred gene expression have been reported for current gene transfer technology into epithelial cells. As demonstrated in this report, another potential therapeutic option is to activate CFTR mutants by more traditional pharmacological approaches. In addition to the mutants, this approach of maximizing CFTR activity may also be necessary to maximally activate wild-type CFTR in gene therapy protocols to compensate for low levels of gene transfer.

It is clear that many disease-causing CFTR mutants can be activated by influencing various parts of the cAMP–PKA pathway. High concentrations of IBMX in conjunction with adenylate cyclase activators have been used to activate a wide range of CFTR mutants in *Xenopus* oocytes (19), phosphatase inhibitors have been used to activate CFTR mutants in chinese hamster ovary cells (14), and increasing PKA activity stimulates CFTR variants associated with mild disease (15, 16). It has also been reported that ΔF508 can be activated through mechanisms other than cAMP–PKA (24, 39, 40). For instance, the substituted benzimidazolone compound NS004 was reported to act directly on CFTR to bring about channel opening in Vero cells (24). In those studies, however, NS004 stimulated activation of ΔF508 CFTR only when used with cAMP or cAMP-elevating agents. The cAMP requirement for ΔF508 CFTR activation suggests that another explanation for NS004 is through phosphodiesterase inhibition, as this compound has a structure similar to the xanthine PDE inhibitors. Regardless of the actual mechanisms involved, all of these reports demonstrate residual function of CFTR mutants. However, expression systems that produce quantities of CFTR vastly greater than those found in vivo (24, 39), and/or use lower incubation temperatures to improve ΔF508 CFTR processing (24) were used. Consequently, it is unclear whether these results are applicable to native epithelia as would be found in a CF patient. Schwiebert et al. (40) found that compounds that inhibit G proteins of the G_i class increase basal chloride conductance with properties consistent with CFTR in transformed airway cells and in primary ΔF508 homozygous CF nasal polyp cells. These studies suggest that endogenous levels of ΔF508 CFTR are sufficient to generate a significant chloride conductance.

In this report, we show that the combination of β agonist and cGI-PDE inhibitors are sufficient to induce CFTR-mediated chloride fluxes in CF airway epithelial cells homozygous for the ΔF508 mutation. Stimulation of chloride transport was found to be dependent on the synergistic action of a β-adrenoreceptor agonist and cGI-PDE inhibitor, and to require PKA activity.

It is noteworthy that the cAMP data (Fig. 3) present an apparent contradiction; the combination of drugs that give the greatest elevations in cAMP are not those that give the greatest increases in efflux. These results suggest that either milrinone and amrinone are affecting chloride transport through a cAMP-independent pathway, or that total cellular cAMP is not the relevant variable to measure. Because the CF cells require both β agonist and phosphodiesterase inhibitor to increase chloride efflux, it is unlikely that a cAMP-independent pathway is responsible. An alternative explanation for the dis-

crepancies between cAMP levels and chloride efflux would be compartmentalization of phosphodiesterase activity as we suggested previously (19). In monocytes, type IV PDE activity is much greater than type cGI-PDE activity, and appears to be limited to the cytosol. In contrast, the relatively small amount of cGI-PDE activity is localized to the particulate fraction (41). Similarly, it has been reported that cGI-PDE is localized to membrane fractions of airway epithelial cells (42). A model consistent with these reports and the data presented here is that the cGI-PDEs, which make up a relatively small part of the total cellular PDE activity in airway epithelial cells, play a major role in CFTR activation due to their membrane localization.

In addition, studies using the calcium chelator BAPTA demonstrate that changes in intracellular calcium activity do not contribute to the observed stimulation of chloride efflux, while the antisense experiments indicate the involvement of CFTR.

Although it remains to be determined what level of activation is needed to have clinical impact, the ability to activate endogenously expressed ΔF508 CFTR in cells incubated at 37°C by influencing its natural regulatory pathway is an important first step in the development of an effective therapy. Optimal activation of CFTR may serve to increase the impact of other potential therapies as well, including gene transfer therapy. If transfer or expression efficiencies remain low, a pharmacological approach to activate CFTR would be beneficial to augment the effectiveness of gene transfer therapy. It is also likely that strategies to improve processing and delivery of the mutants to the plasma membrane will eventually be realized, increasing the potential effectiveness of this approach. Whether used alone or in conjunction with other treatments, the ability to increase chloride transport in CF cells through pharmacological activation of mutant CFTR is a potentially powerful asset to future CF therapies.

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