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Apical Heterotrimeric G-proteins Activate CFTR in the Native Sweat Duct

M.M. Reddy, D. Sun, P.M. Quinton
University of California, San Diego, School of Medicine, Department of Pediatrics-0831, La Jolla, CA 92093-0831, USA

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Abstract. Other than the fact that the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel can be activated by cAMP dependent kinase (PKA), little is known about the signal transduction pathways regulating CFTR. Since G-proteins play a principal role in signal transduction regulating several ion channels [4, 5, 9], we sought to test whether G-proteins control CFTR Cl^- conductance (CFTR G_{Cl}) in the native sweat duct (SD). We permeabilized the basolateral membrane with α-toxin so as to manipulate cytosolic nucleotides. We activated G-proteins and monitored CFTR G_{Cl} activity as described earlier [20, 23, 25]. We now show that activating G-proteins with GTP-γ-S (100 μM) also activates CFTR G_{Cl} in the presence of 5 mM ATP alone (without exogenous cAMP). GTP-γ-S increased CFTR G_{Cl} by 44 ± 20 mS/cm^2 (mean ± SE; $n = 7$). GDP (10 mM) inhibited G-protein activation of CFTR G_{Cl} even in the presence of GTP-γ-S. The heterotrimeric G-protein activator (AlF_4^-) in the cytoplasmic bath activated CFTR G_{Cl} (increased by 51.5 ± 9.4 mS/cm^2 in the presence of 5 mM ATP without cAMP, $n = 6$), the magnitude of which was similar to that induced by GTP-γ-S. Employing immunocytochemical-labeling techniques, we localized Gαs, Gαi, Gαq, and Gβ at the apical membranes of the sweat duct. Further, we showed that the mutant CFTR G_{Cl} in ducts from cystic fibrosis (CF) subjects could be partially activated by G-proteins. The magnitude of mutant CFTR G_{Cl} activation by G-proteins was smaller as compared to non-CF ducts but comparable to that induced by cAMP in CF ducts. We conclude that heterotrimeric G-proteins are present in the apical membrane of the native human sweat duct which may help regulate salt absorption by controlling CFTR G_{Cl} activity.

Correspondence to: P.M. Quinton

Key words: cAMP — GTP-γ-S — ENaC — Na/K ATPase — Cystic Fibrosis

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl^- channel [18, 29, 33] well known to be activated by cAMP dependent protein kinase (PKA). Although other means of phosphorylating and thereby activating the channel have been implicated, little is known of the physiological pathways that regulate this channel and its attendant fluid transport functions [18, 29, 33]. Abnormalities in CFTR may cause life threatening pathologies by expressing either abnormally low (cystic fibrosis, CF) or abnormally high (secretory diarrhea) Cl^- conductances [7, 18, 29]. A better understanding of the physiological signal transduction pathway regulating this important anion channel may be key to developing effective pharmacological interventions. Moreover, the process of acutely regulating electrolyte absorption seems relatively unappreciated and poorly studied as compared to regulation of electrolyte secretion. Since the predominant function of the SD is to absorb NaCl from the primary sweat secreted by the sweat secretory coil [18, 19, 23], the SD offers a good opportunity to study physiological signal transduction mechanisms involved in regulating CFTR in a native absorptive tissue.

G-proteins play a significant role in ligand activated signal transduction mechanisms leading to activation/deactivation of different ion channels [4, 5, 9]. In view of this perspective, we questioned whether G-protein might play a role in regulating the CFTR G_{Cl} of the SD. Several epithelial cells expressing CFTR also express G-proteins strategically located in the apical membranes [30–31]. However, nothing is known about G-protein

regulation of CFTR in a native human absorptive epithelium.

G-proteins are a family of membrane-bound proteins that exist in both monomeric and heterotrimeric forms. The heterotrimeric G-proteins are made up of α, β, and γ sub-units. Many gene products are recognized to encode each class of subunits of G-proteins. Three heterotrimeric G-proteins are best characterized on the basis of general functions which include: Gαs, commonly activates adenylyl cyclases (AC); Gαi; commonly inhibits AC; Gαq, commonly activates phospholipase C [12]. GDP bound G-proteins are inactive. G-proteins may regulate ion channels by different mechanisms including: regulation of AC/cAMP/PKA cascade dependent phosphorylation, control of PKC dependent phosphorylation through inositol phosphate metabolites including inositol triphosphates (IP_3; hence intracellular Ca^{2+}) and diacylgycerol, and via direct interaction with channel proteins [4, 5, 12]. Since CFTR can be activated pharmacologically by activating both PKA and PKC, it seems important to know whether G-proteins are involved in its control. Therefore, we attempted to functionally characterize and immunocytochemically localize different G-proteins in the sweat duct.

We sought to determine whether: (i) CFTR can be activated by stimulating G-proteins in native absorptive cells, (ii) heterotrimeric G-proteins can activate CFTR, (iii) G-proteins are present in the apical membrane of duct cells, and (iv) G-protein stimulation might offer another approach for regulating mutant CFTR in CF duct cells.

Materials and Methods

Tissue Acquisition

Sweat glands were obtained from adult male volunteers without medical history who gave informed consent. Individual sweat glands were isolated from the skin in Ringer's solution (maintained at ~5°C) by dissection with fine tipped tweezers under a dissection microscope. The isolated glands were transferred to a cuvette with Ringer's solution cooled to 5°C where the segments of reabsorptive duct (~1 mm in length) were separated from the secretory coil of the sweat gland under a dissecting microscope (Nikon model SMZ-10). Using a glass micropipette (ca 200 nL), the sweat duct was transferred to a perfusion chamber containing Ringer's solution for cannulation and microperfusion at 35 ± 2°C.

Selective Permeabilization of the Basolateral Membrane

The basolateral membrane of the sweat duct was selectively permeabilized with a pore-forming agent (1,000 units of α-toxin derived from *Staphylococcus aureus*) in cytoplasmic Ringer's solution containing 140 mM KGlu (potassium gluconate) and 5 mM ATP applied to the basolateral surface of the microperfused sweat duct for 15 to 30 min. As described earlier, α-toxin effectively removes the basolateral membrane as a barrier to cAMP and ATP apparently without affecting the functional integrity of the apical membrane. This preparation allows free manipulation of intracellular cAMP, ATP, GTP and similar nucleotides so that the properties of the regulation of CFTR-GCl in the apical membranes can be studied in relative isolation [21, 23].

Electrical Measurements

After cannulating the lumen of the sweat duct with a double lumen cannula made from theta glass, a constant current pulse of 50–100 nA for a duration of 0.5 sec was injected through one barrel of the cannulating pipette containing NaCl Ringer's solution. The other barrel of the cannulating pipette served as an electrode for measuring transepithelial potential (V_t) with respect to the contraluminal bath and as a cannula for perfusing the lumen of the duct with selected solutions. V_t was monitored continuously using one channel of WPI-700 dual electrometer referenced to the contraluminal bath. Transepithelial conductance (G_t) was measured as described earlier [10, 21] from the amplitude of transepithelial voltage deflections in response to transepithelial constant current pulse using the cable equation.

Cl^- diffusion potentials (V_{Cl}) and G_{Cl} were monitored to indicate the level of activation of G_{Cl}. Following α-toxin permeabilization of the basolateral membrane, the epithelium is simplified to a single (apical) membrane with parallel Na^+ and Cl^- conductances. Application of amiloride further simplified the system a predominantly Cl^--selective membrane. The composition of Ringer's solution in bath and lumen was designed to set up a single ion gradient, i.e., exclusively for Cl^- [140 mM K gluconate (bath)/150 mM NaCl (lumen)]. Under these conditions V_t and G_t can be regarded essentially as V_{Cl} and G_{Cl} respectively [21, 23, 24].

Immunohistochemistry

Skin plugs were fixed overnight in 2% paraformaldehyde and transferred to 30% sucrose overnight for cryoprotection. The tissue was then imbedded in OCT 4583 compound and stored at −80°C. 5–8 μM sections were cut with a microtome (Leica CM3000-Cryostat) and mounted on slides. The slides were stored at −20°C. Endogenous peroxidase was blocked by exposure to 0.3% H_2O_2 for 15 min at room temperature. The slide was then washed three times with PBS, each time for 10 min. Nonspecific staining was blocked by pretreatment with 10% normal goat serum in PBS (phosphate buffered saline) for 1 hr at room temperature. Sections were incubated at 4°C overnight with primary antibodies for either for Gαs, Gαi, Gαq or Gβ (Santa Cruz Biotechnology, catalog #sc-823, sc263, sc393, sc378, respectively). Antibody (1 μl) was mixed with 0.4 to 1 ml of PBS containing 0.3% Triton with and without the respective blocker peptide. Negative controls were prepared by incubating sections in rabbit IgG (same concentration as that of primary antibody) without the primary antibody. Sections were then washed three times (10 min each) in PBS. The primary antibody was detected with biotinylated goat anti-rabbit IgG using an immunoperoxidase reaction (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Antibodies were visualized by using diaminobenzidine as the substrate for peroxidase color development. The sections were then dehydrated and coverslips were permanently mounted. The specificity of immunolabeling was confirmed by controls using incubation: (i) without primary antibody, (ii) without secondary antibody, and (iii) with fusion peptide [34]. Additional positive

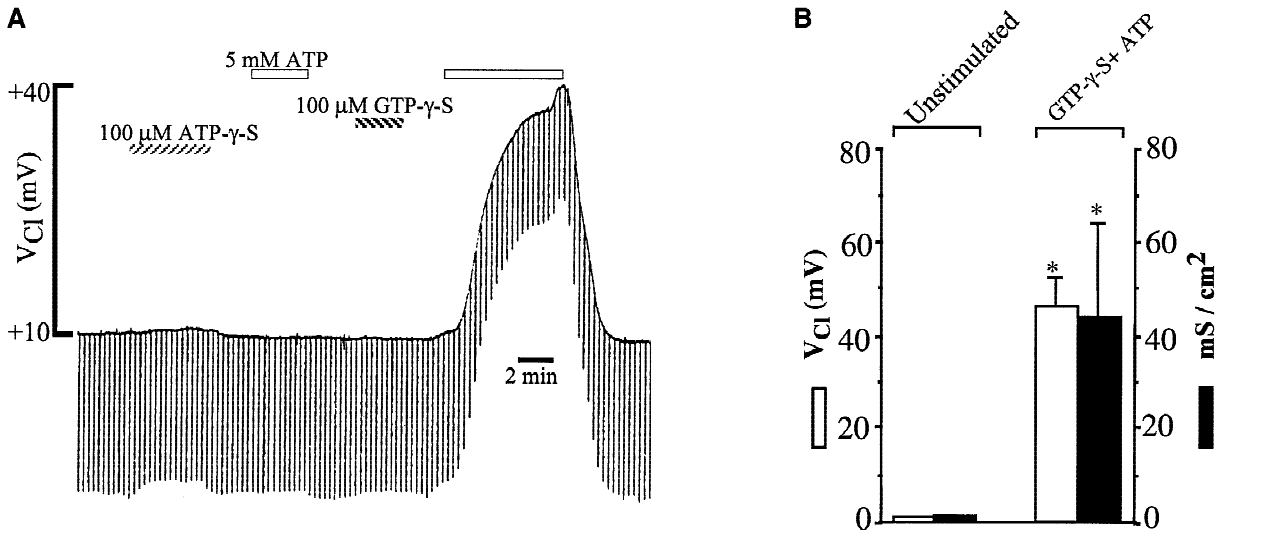

Fig. 1. The effect of GTP-γ-S on ATP activation of CFTR. (*A*) In this experiment GTP-γ-S was applied to the cytoplasmic side in the complete absence of ATP. Excess GTP-γ-S (not bound to the G-proteins) in the bath was washed out. Under these conditions subsequent application of 5 mM ATP activated CFTR G_{Cl} independent of cAMP. This effect of GTP-γ-S was not mimicked by ATP-γ-S indicating that phosphatase resistant thiophosphorylation of CFTR was not responsible for the observed results. These results indicate that CFTR G_{Cl} is regulated by G-proteins in the duct. (*B*) Summary of the data collected from similar experiments as shown in the left panel. Notice that after G-protein activation, ATP alone stimulated CFTR G_{Cl} as indicated by the significant increase in Cl^- diffusion potential (V_{Cl}) Cl and conductance (CFTR G_{Cl}), which are comparable to those induced by cAMP + ATP before activating the G-proteins ($n = 7$, $P < 0.001$).

controls for the immunohistochemistry protocol was provided by membrane specific labeling of amiloride sensitive epithelial Na^+ channels (ENaC) in the apical membrane and Na-K ATPase in the basolateral membrane with their respective antibodies (Fig. 6*b*). ENaC and Na-K ATPase were labeled with anti-β human ENaC (courtesy of Dr. D. Benos and Dr. C. Fuller) and anti-chicken α-subunit of Na-K ATPase (Hybridoma bank).

Solutions

The luminal perfusion Ringer's solutions contained (in mM): NaCl (150), K (5), PO_4 (3.5), $MgSO_4$ (1.2), Ca^{2+} (1), and amiloride (0.01), pH 7.4. Cl^--free luminal Ringer's solution was prepared by substitution of Cl^- with impermeant anion gluconate. The cytoplasmic/bath solution contained K (145), gluconate (140), PO_4 (3.5), $MgSO_4$ (1.2), and 260 μM Ca^{2+} buffered with 2.0 mM EGTA (Sigma) to 80 nM free Ca^{2+}, pH 6.8. K^+ free cytoplasmic bath solution was prepared by complete substitution of equimolar concentration of K^+ with Na^+. K^+ ATP (5), ATP-γ-S (5) and cAMP (0.1), GTP (1 mM), GTP-γ-S (0.1), GDP (10), $AlCl_3$ (0.1), KF (5) and vanadate (1 mM) were added to the cytoplasmic bath as needed. Na^+ATP was used in K^+ free cytoplasmic bath solution.

Data Analysis

The V_{Cl} and G_{Cl} in the bar figures represent peak values which were stable for at least 2 min within ±2 mV. The data is presented as the mean ± SE (where n = number of ducts from a minimum of 4 human subjects). Statistical significance was determined on the basis of Student's t test for paired samples. A 'P value' of <0.05 was taken as significantly different. Data when presented as representative examples of similar experiments were repeated at least three times.

Results

Effect of GTP-γ-S

After α-toxin permeabilization of the basolateral membrane of the sweat duct, the cytoplasmic nucleotides responsible for phosphorylation activation of CFTR leak out of the cell so that CFTR spontaneously deactivates. Deactivation of CFTR is indicated by the loss of G_{Cl} and V_{Cl} across the apical membrane. Conventionally, reactivation of CFTR requires the presence of both 0.1 mM cAMP + 5 mM ATP in the cytoplasmic bath. Removing cAMP deactivates CFTR even in the presence of ATP indicating spontaneous dephosphorylation of CFTR by endogenous phosphatases. However, after adding 10 to 100 μM GTP-γ-S to the cytoplasmic bath, CFTR was activated by ATP alone, without cAMP, as indicated by significant increases in both G_{Cl} and V_{Cl} (Fig. 1*A*; $\Delta G_{Cl} = 44.1 \pm 19.5$ mS/cm^2 and $\Delta V_{Cl} = 46.4 \pm 4.8$ mV; mean ± SE, n = number of ducts = 7, $P < 0.001$). The effect of GTP-γ-S on CFTR was irreversible for the duration of the experiment (>1 hr). The effect of G-protein activation on CFTR G_{Cl} was comparable to that of 0.1 mM cAMP + 5 mM ATP (Fig. 1*b*). To determine the dose response, we tested the effect of 1, 10 and 100 μM GTP-γ-S on CFTR G_{Cl}. While 1 μM GTP-γ-S had little effect (*results not shown*), no significant difference was found between the stimulating effects of 10 and 100 μM GTP-γ-S on CFTR G_{Cl}. Therefore, in later experiments we used 10 μM GTP-γ-S to stimulate CFTR G_{Cl}.

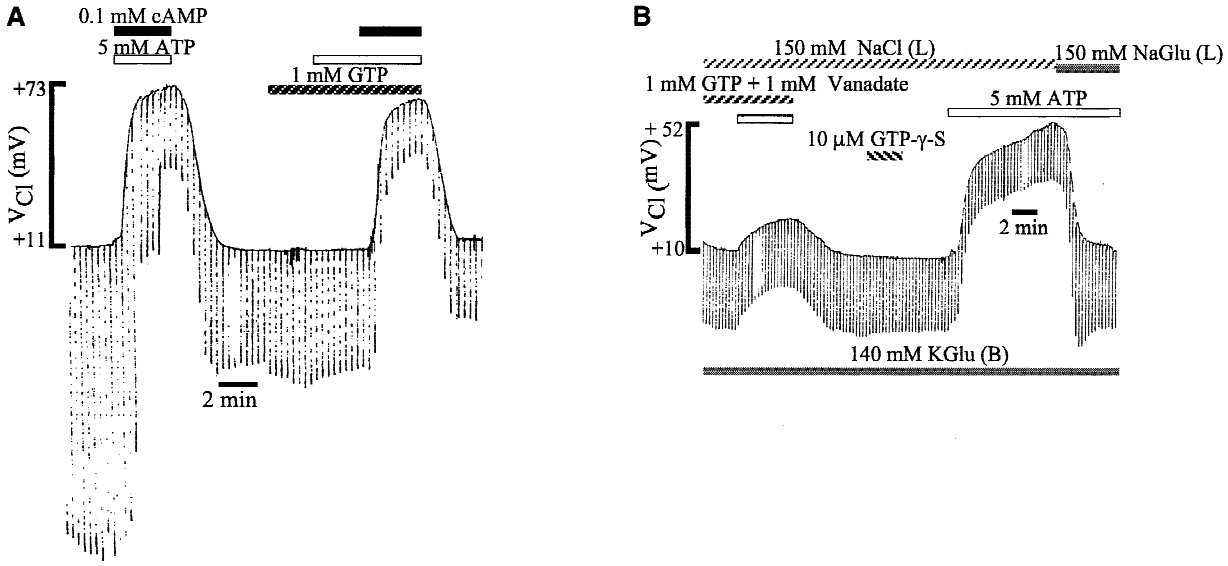

Fig. 2. The effect of hydrolyzable GTP on G-proteins. (*A*) This experiment tested the effect of GTP (1 mM) on G-protein/CFTR G_{Cl} activation in the presence of ATP (5 mM). Notice that hydrolyzable GTP neither activated CFTR G_{Cl} (hence no activation of G-proteins) nor did it affect the magnitude of CFTR G_{Cl} activation by cAMP in the presence of ATP. (*B*) We tested the effect of inhibiting GTPase activity with vanadate (1 mM) on GTP activation of G-proteins and CFTR G_{Cl}. Notice that GTP partially activated CFTR G_{Cl} in the presence of vanadate and ATP, even though the activation was small compared to that induced by GTP-γ-S. Also note that the electrical effects induced by GTP-γ-S (i.e., depolarization and increased conductance) was abolished in the absence of a Cl^- gradient (the impermeable anion gluconate was substituted for luminal Cl^-).

Effect of Physiological GTP

Addition of physiological GTP to the cytoplasmic bath did not stimulate G-proteins as indicated by a lack of CFTR G_{Cl} activation in the presence of 5 mM ATP + 1 mM GTP (Fig. 2*A*). However, partial activation of CFTR G_{Cl} was detectable after inhibiting GTPase activity with 1 mM vanadate in the cytoplasmic bath. Such activation was significantly smaller as compared with that induced by GTP-γ-S. The effect of GTP-γ-S on transapical conductance and potential was abolished when the Cl^- gradient was removed by complete Cl^- substitution in the lumen by impermeable anion gluconate (Fig. 2*B*).

Effect of GDP on G-Protein Activation

Application of GDP (10 mM) prevented GTP-γ-S activation of G-proteins as indicated by a lack of activation of CFTR G_{Cl} when ATP was added to the cytoplasmic bath. Nonetheless, in the same duct, GTP-γ-S activated CFTR G_{Cl} (in the presence of ATP) after washing out GDP (Fig. 3).

Effect of AlF_4^-

Similar to the effect of GTP-γ-S, application of an agonist cocktail containing 100 μM $AlCl_3$+ 5 mM KF to activate heterotrimeric G-proteins also activated CFTR in the presence of ATP alone without cAMP (Fig. 4). In the presence of ATP, the G-protein agonist cocktail increased CFTR G_{Cl} and V_{Cl} by 51.7 ± 9.3 mS/cm^2 and 49.5 ± 7.3 mV respectively ($n = 6$, $P < 0.001$). Unlike the effect of GTP-γ-S, the effect of AlF_4^- was fully reversible so that after washing out AlF_4^-, ATP alone could not activate CFTR without cAMP. The effect of AlF_4^- on CFTR G_{Cl} was also comparable to that of cAMP + ATP in the same ducts, but it did not stimulate CFTR G_{Cl} in the absence of ATP (Fig. 4). To test whether $AlCl_3$ or KF had an effect on CFTR when applied separately, we applied each compound to the cytoplasmic bath individually in the presence and absence of ATP. Neither $AlCl_3$ nor KF applied separately had any effect on CFTR in the presence or absence of ATP (Fig. 5).

Immunohistochemical Localization of G-Proteins

Antibodies against Gαs, Gαi, Gαq, and Gβ were localized in both the apical and basolateral membranes of sweat duct. In the presence of fusion peptide or in the absence of primary antibody, the labeling was scant or absent indicating that G-protein labeling was specific for these heterotrimeric G-protein subunits (Fig. 6*a*).

Membrane Specific Localization of ENaC and Na-K ATPase

As controls for the immunohistochemical labeling protocols, cell membranes were labeled with antibodies for

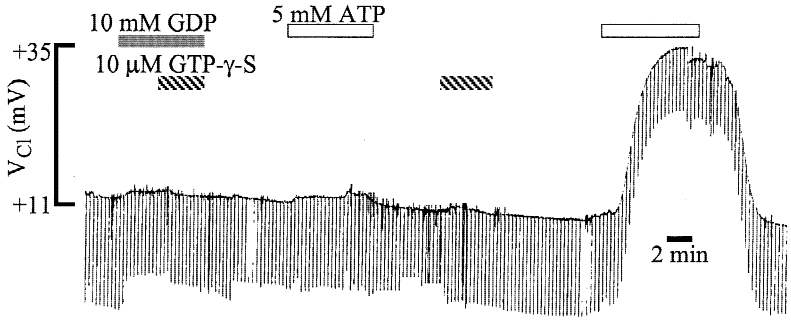

Fig. 3. The effect of GDP on G-protein activation of CFTR G_{Cl}. To test the effect of GDP on GTP-γ-S activation of G-proteins, we first exposed the apical membranes to 10 mM GDP before adding 10 μM GTP-γ-S to the cytoplasmic bath. Notice that ATP alone did not activate CFTR G_{Cl} after GTP-γ-S was applied in the presence of GDP. However, after GTP-γ-S was applied without GDP, ATP activated CFTR G_{Cl} in the same ducts.

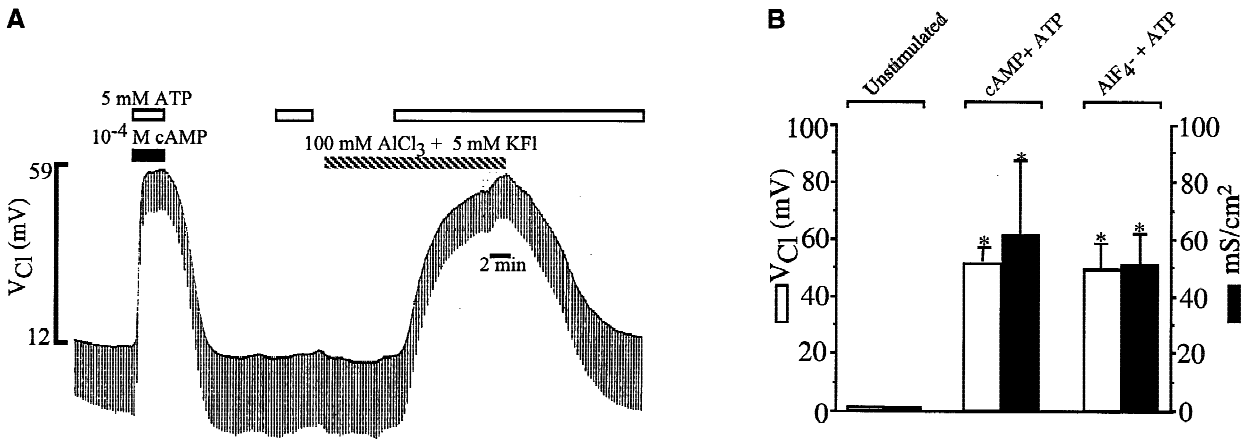

Fig. 4. Effect of AlF_4^- on CFTR G_{Cl}. (*A*) To test whether the monomeric as opposed to trimeric G-proteins are involved in activating CFTR, we applied 100 μM $AlCl_3$ + 5 mM KF to the cytosolic bath of the permeabilized duct to generate the AlF_4^- complex anion. AlF_4^- selectively activates trimeric, but not monomeric, G-proteins. In the presence of AlF_4^-, ATP alone activated CFTR G_{Cl}, but AlF_4^- by itself did not have an effect in the absence of ATP. (*B*) Summary of the data on the effect of AlF_4^- and cAMP + ATP on CFTR G_{Cl} of the same ducts as shown in the left panel. The effect of AlF_4^- on CFTR G_{Cl} was comparable to that of cAMP and ATP ($n = 6$, $P < 0.001$).

well-characterized transport proteins known to be specific for the apical or basolateral membranes. The anti-β subunit of the human epithelial Na^+ channel (ENaC) specifically labeled the apical membrane of the reabsorptive sweat duct (Fig. 6*b*, left panel). Neither the basolateral membrane of the duct nor either membrane of the secretory coil labeled with the ENaC antibody. In contrast, monoclonal anti-chicken α-subunit of Na-K ATPase specifically labeled the basolateral, but not the apical membrane of the duct and the secretory coil (Fig. 6*b*, right panel).

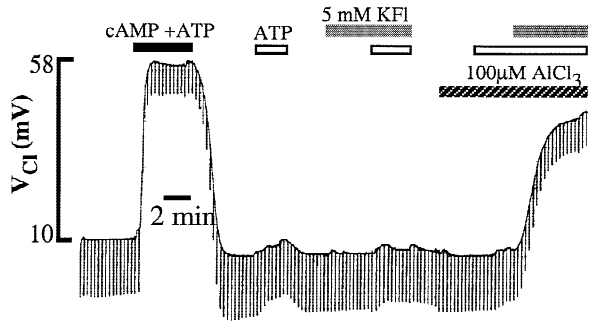

Fig. 5. Both Al^{3+} and F^- are required for G-protein activation. In this experiment we assessed the effect of either Al^{3+} or F^- alone on G-protein activation. G-protein activation was monitored by measuring CFTR G_{Cl} activation with ATP alone after application of either 100 μM $AlCl_3$ or 5 mM KF. ATP alone did not activate CFTR G_{Cl} unless both Al^{3-} and Fl^- were simultaneously present.

Effect of GTP-γ-S on CF Ducts

Application of GTP-γ-S increased mutant CFTR G_{Cl} in a CF duct from a ΔF508 homozygous patient in whom the mutated CFTR protein is known to be poorly processed to the plasmamembrane. Even though the magnitude of CFTR G_{Cl} activation was much smaller in CF than in non-CF ducts, the activation was comparable to that of cAMP + ATP in the same ducts (Fig. 7). As in non-CF ducts the effect of GTP-γ-S was irreversible so that CFTR G_{Cl} remained stimulated as long as ATP was present.

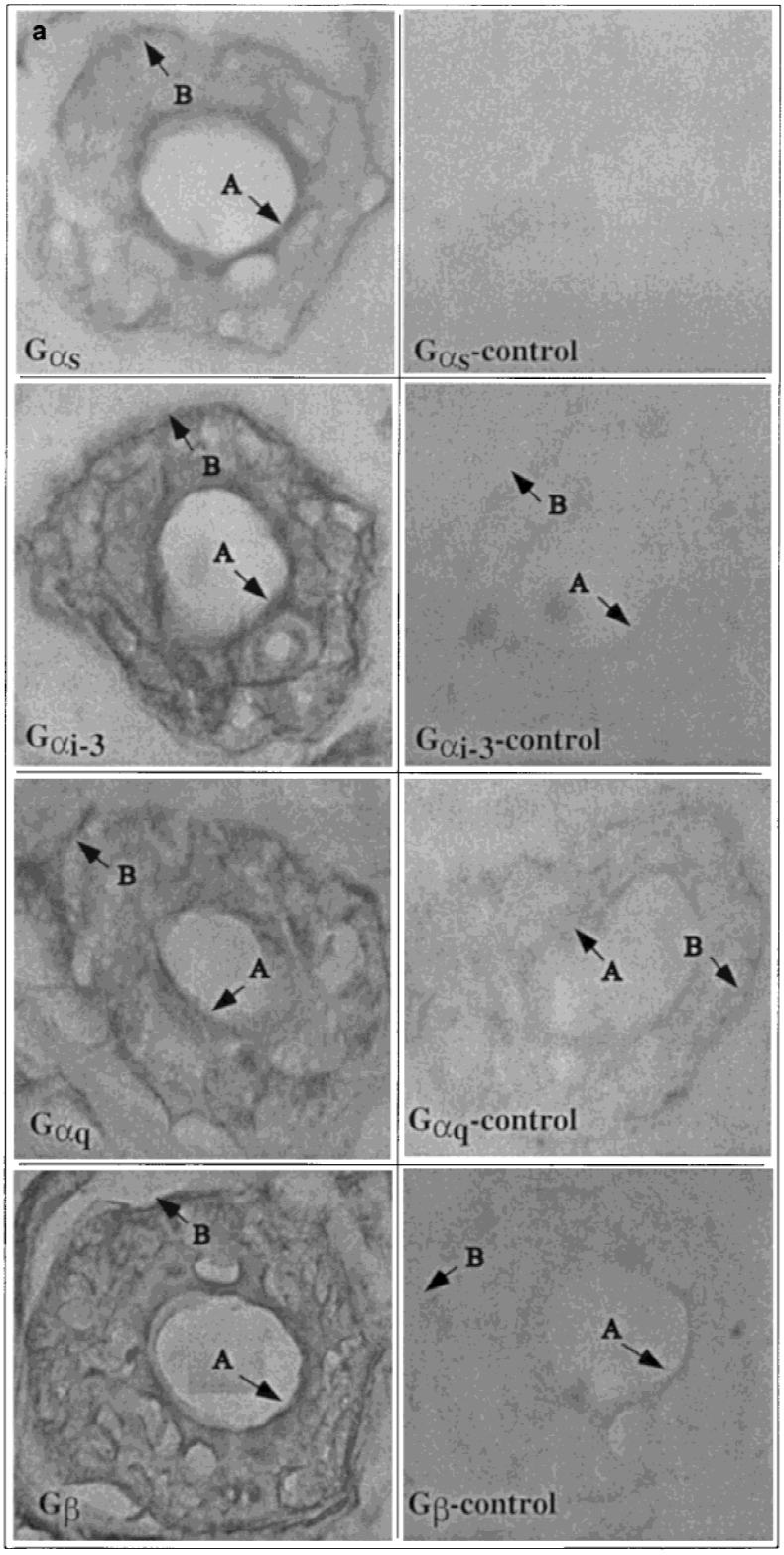

Fig. 6 *(a).* Apical localization of G-proteins. Antibody immunocytochemical localization of Gαs, Gαi, Gαq, and Gβ subunits of heterotrimeric G-proteins in the apical membrane of intact (nonpermeabilized) sweat ducts (left panel top to bottom). The basolateral membranes were also stained by the respective antibodies suggesting that these G-proteins are present in both apical and basolateral membranes. G-protein labeling was almost completely absent in respective controls when the antibody was blocked with the fusion peptide as shown respectively, in the panels to the right of the adjacent figures.

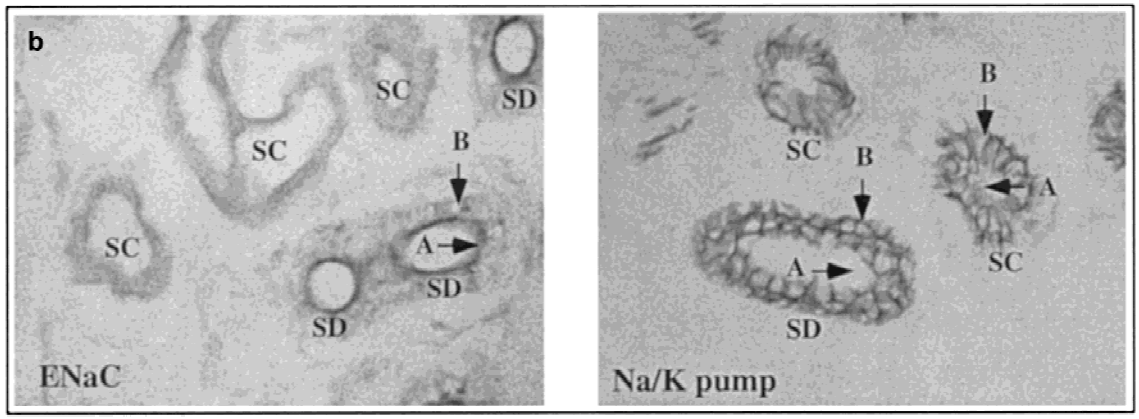

Fig. 6 *(b).* Membrane-specific localization of ENaC and Na-K ATPase. Immunohistochemical labeling of amiloride-sensitive epithelial Na^+ channel (ENaC) with anti-β human ENaC was specifically localized at the apical membrane of the reabsorptive sweat duct (Left panel). As expected neither the basolateral membrane of the duct nor any of the membranes of the secretory coil cell were labeled with the ENaC antibody. Similarly, monoclonal anti-chicken α-subunit of the Na-K ATPase specifically labeled the basolateral, but not the apical membrane of the duct and the secretory coil (right panel).

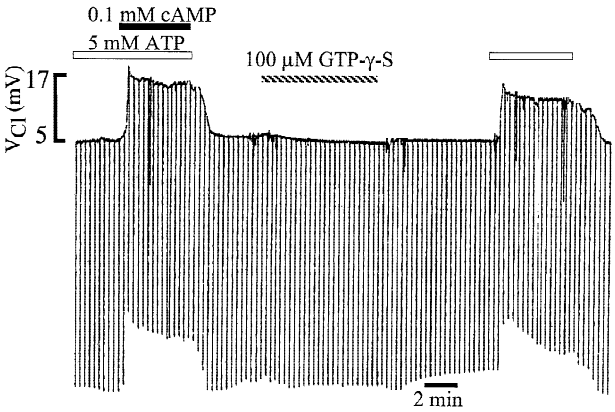

Fig. 7. G-protein activation of mutant CFTR from a CF duct. Activating G-proteins with GTP-γ-S resulted in activation of mutant CFTR G_{Cl} by ATP in a duct from a CF patient homozygous for ΔF508. Notice that before activating G-proteins, ATP had little effect on the mutant CFTR. These results indicated that the G-proteins could activate certain mutant forms of CFTR, which might be exploited to pharmacologically enhance the G_{Cl} activity in CF epithelial membrane.

Discussion

Although G-proteins play a significant role in signal transduction involved in epithelial ion channel regulation [4, 5, 9, 30, 31], little is known of their role in controlling the highly activatable CFTR chloride channel. In this study we sought to determine if we could find evidence of G-protein regulation of this channel. We therefore determined to apply some of the criteria used in characterizing physiological roles for heterotrimeric G-proteins in ion channel regulation. We examined the following requirements: (i) the nonhydrolyzable GTP analogue GTP-γ-S must influence ion channel function, (ii) the effect of GTP-γ-S should be mimicked by AlF_4^-, (iii) the effect of G-protein activation on ion channel function should be inhibited or reversed by GDP, and (iv) the trimeric G-proteins should be localized in the plasma membrane [4, 9, 15, 27].

Heterotrimeric G-proteins Can Activate CFTR G_{Cl}

As shown in Fig. 1 application of a low concentration of GTP-γ-S (10–100 μM) in the presence of 5 mM ATP significantly increased apical membrane conductance. The increase in apical electrical conductance by GTP-γ-S seems to be entirely due to activation of apical CFTR G_{Cl} because: (i) the conductance is sensitive to cytosolic ATP (Fig. 1); (ii) the GTP-γ-S induced changes in depolarizing potentials and in increased conductance across the apical membrane are abolished when the Cl^- gradient is removed (by substituting luminal Cl^- with the impermeable, anion gluconate). Hence, it is highly unlikely that GTP-γ-S activates an amiloride insensitive K^+-selective apical cation channel, which is the only other source of such electrical signals (Fig. 2), (iii) the anion conductance across the apical membrane is almost exclusively comprised of CFTR [20, 23]; and (iv) stimulation with either GTP-γ-S or cAMP yields quantitatively similar responses and, the responses are not additive. Although it seems clear that GTP-γ-S is activating CFTR G_{Cl}, it is nonetheless possible that the action is not through a G-protein. That is, it is possible that GTP-γ-S might thiophosphorylate CFTR. The thio ester is resistant to phosphatase dephosphorylation as shown previously when ATP-γ-S was used as the phosphorylation

substrate [23–25]. If CFTR were irreversibly phosphorylated by an endogenous kinase using GTP-γ-S as substrate, addition of ATP would activate CFTR G_{Cl} without cAMP [23–25]. To exclude this possibility, we compared the effect of equimolar concentrations of GTP-γ-S and ATP-γ-S on CFTR G_{Cl} activation in the presence of 5 mM ATP. The fact that ATP did not activate CFTR G_{Cl} in ducts previously exposed to ATP-γ-S (Fig. 1) indicated that action of GTP-γ-S is not merely due to thiophosphorylation of CFTR, but most likely due to the activation of G-proteins.

Effect of GTP

We further tested the effects of GTP itself to determine whether the native form of this nucleotide might activate CFTR. The rate of GTP-hydrolysis determines the duration of the Gα subunit protein in its active state [4, 9, 15, 30]. Cells normally contain ~100 μM GTP and 10 μM GDP, and Gα generally shows high affinity GTP binding (K_m ~ 0.3 μM) [4] so that the cytoplasmic pool of GTP for binding upon receptor activation is supersaturating. We applied a 10-fold higher concentration of GTP (1 mM) in the complete absence of its competitive inhibitor GDP, but as indicated by a complete lack of CFTR G_{Cl} activation when ATP was added, the G-protein apparently failed to activate. However, addition of GTP after inhibiting GTPase activity with vanadate (1 mM) resulted in partial activation of CFTR G_{Cl} (Fig. 4), which is consistent with earlier observations on Cl^- channels in renal [31] and intestinal epithelial cells [3, 8, 35]. This observation suggests that the GTPase activity of G-proteins in the permeabilized duct is high and that GTP hydrolysis is too rapid to effect CFTR activation without inhibiting the GTPase [1, 28]. Nonetheless, this supports the notion that CFTR can be activated by a G-protein.

Mutant CFTR

The finding that G-protein stimulation is a new, highly effective means of activating CFTR suggested that it might be used to activate mutant forms of the channel protein. The fat that we were able to induce a small CFTR G_{Cl} via activation of a G-protein may offer some promise of another avenue for ameliorating the effects of CF. In this particular case, the ΔF508 mutant is known to be poorly processed, but to retain some function when expressed in the plasma membrane [6]. Thus, if appropriate G-protein receptors can be identified to couple activation of CFTR, specific stimulation of these receptors might prove beneficial to restoring some level of function in cells expressing this class of mutations.

Heterotrimeric vs. Monomeric G-proteins

Since GTP-γ-S can activate both monomeric and heterotrimeric G-proteins, it was necessary to exclude the possibility that the activation is not proceeding through small, monomeric GTPases. AlF_4^- is thought to specifically activate heterotrimeric G-proteins and is often used for this purpose [16]. Micromolar concentrations of aluminum facilitate fluoride stimulation of G-proteins, probably due to formation of an AlF_4^- complex that mimics GTP binding but cannot be hydrolyzed [2, 16, 27]. Figure 5 shows that AlF_4^- activates CFTR G_{Cl} in a manner and magnitude similar to that induced by either cAMP or GTP-γ-S. Nonetheless, aluminum is known to have diverse effects on several enzyme systems, such as inhibition of PKC along with associated decreases in phosphoinositide hydrolysis and IP_3 accumulation [14, 32]. Similarly, F^- is known to inhibit endogenous phosphatases and therefore might prevent dephosphorylation and deactivation of CFTR [24]. To ensure that the observed effects of $AlCl_3$ and KF were in fact due to AlF_4^- complex, we added $AlCl_3$ and KF separately as well as simultaneously. ATP activated CFTR only when Al^{3+} and F^- were present simultaneously (Fig. 6) indicating that the AlF_4^- complex is required and responsible for G-protein activation of CFTR. Thus, we conclude that the G-protein that regulates CFTR in this epithelium is most likely heterotrimeric.

GDP Inhibits the G-Protein Activation

Since GDP inhibition of activated G-proteins is a characteristic property of this process [4, 15, 30], we tested the effect of GDP on GTP-γ-S activation of CFTR G_{Cl}. We exposed the duct to GTP-γ-S in the presence and absence of GDP (10 mM) and tested the ability of ATP alone to activate CFTR G_{Cl}. As shown in Fig. 3, ATP activated CFTR G_{Cl} only after the duct was exposed to GTP-γ-S in the absence of GDP. This result suggests that GDP competitively inhibits GTP binding, providing further evidence that G-proteins are involved in regulating CFTR.

Localization of the G-Proteins

The role of apical G-proteins in regulating ion channels and transport activity in the apical membrane in the sweat duct is unknown. However, two principle elements that play a central role in transepithelial salt absorption, namely CFTR Cl^- channels and amiloride-

sensitive ENaC channels, are strategically located in the apical membrane [18, 21]. We know that the apical membrane is exposed to changes in salt loads in the lumen (and therefore luminal salt concentrations) as a function of gland secretory rate. It seems likely therefore that if signal transduction mechanisms are an important part of this process, G-proteins might also be strategically positioned to acutely respond to the luminal environment. Therefore, we tested for the expression of G-protein subunits in the apical membranes of sweat ducts.

Since all trimeric G-proteins are composed of three subunits: α, β, and γ, in which the α subunit gives specificity to the protein function and the βγ dimer is common to, or very similar in all heterotrimers, expression of the βγ subunit is perquisite to demonstrating the presence of any trimeric complex. We tested for the presence of the β subunit immunocytochemically with an antibody raised against the carboxy terminus of $G_{\beta 1}$ of mouse origin. Most cells express more than one family, possibly numerous families, of G-proteins [12] so that while demonstration of the presence of a common subunit, e.g., *G*β, in the sweat duct does not give any information as to specific function, it is a condition necessary for demonstrating the presence of G-proteins. Figure 6*a* shows labels of both plasma membranes and probably of cytosolic structures as well.

We then tested for three α subunits of common families of heterotrimers to determine if we could reduce the possibilities of families that might be candidates for regulating CFTR in the apical membrane. All three subunits appear to be expressed. The Gαi,3 and Gαq seemed to be expressed more or less equally at both membranes and possibly in cytoplasmic structures (Fig. 6*a*). However, labels for Gαi,3 seemed to be much more distinct than Gαq with respect to labeling both the luminal and basolateral membranes. While it is not possible to make quantitative judgments about levels of expression from these assays, the more diffuse and possibly weaker staining for Gαq may suggest a less pronounced role for this family in ductal CFTR function. On the other hand, the fact that CFTR is highly expressed in the apical membrane and the clear, relatively dominant staining of the apical membrane of the duct for Gαs could implicate this family of G-proteins in the regulation of apical membrane transport processes and control of CFTR. If this protein is linked to control of CFTR in microdomains of the membrane [17], the less intense staining of the basolateral membrane seems consistent with the much weaker staining of CFTR in this membrane [13]. Furthermore, the Gαs family is commonly associated with control of adenylyl cyclase and therefore cAMP. The fact that CFTR is well known to be stimulated by cAMP-activated protein kinase (PKA) is also consistent with a role for this G-protein in controlling CFTR.

The immunohistochemical localization of antibodies in the apical and basolateral membranes is well characterized, Na/K ATPase (generally found only in the basolateral membrane) and ENaC (known to be highly expressed in the apical membrane of the duct) not only shows that the immunocytochemical protocol used here is effective, but also gives some basis for comparison for specific membrane labeling. Although the three Gα subunits appear to be present in the basolateral membrane, it seems unlikely that the G-proteins in the basolateral membrane are responsible for CFTR G_{Cl} activation in the apical membranes of permeabilized ducts. Although it is conceivable that basolateral G-proteins might regulate apical CFTR via intracellular messengers such as cyclic nucleotides or Ca^{2+}, GTP-γ-S and $AlFl_4^-$ apparently can activate CFTR in the complete absence of cytosolic cyclic nucleotides and Ca^{2+} in the sweat duct [26]. Since α-toxin treatment functionally obliterates the basolateral membrane and seems to prevent accumulation of cytosolic nucleotides, we surmise that the G-protein regulation of CFTR in the duct is likely to be a membrane delimited phenomenon such that activation of the kinase and CFTR take place in a very localized domain [17].

What Activates the G-protein?

The finding that G-proteins can activate CFTR immediately raises the question of what activates the G-protein. Generally, ligand binding to G-protein coupled receptors is the initial step in the dissociation of GDP from Gα, and subsequent GTP binding which activates the Gα-subunit [4, 5, 9, 16]. Primary sweat from the secretory coil might contain such a ligand to apical receptors in the duct, which would activate CFTR and salt absorption. However, in the perfused duct, the perfusion solution is a completely defined Ringer's solution with no organic constituents other than glucose. Apically applied purinergic receptor agonists AMP, ATP and UTP all failed to increase CFTR G_{Cl} in intact nonpermeabilized sweat ducts [22; *personal observations*], suggesting that these agonists do not activate apical G-protein coupled receptors in the sweat duct. These results raise the suspicion that G-proteins of the apical membrane might be mechanically activated as suggested for human endothelial cells [11]. Microperfusion, as does physiological sweating, unavoidably dilates the lumen and may "stretch" the apical membrane. Further studies are needed to determine what effect, if any, such physical phenomenon may have on G-protein mediated activation of CFTR G_{Cl}. However, assays of apical membrane function without dilation of the lumen present a formidable challenge.

CFTR and G-proteins in Other Systems

Early studies on cultured non-CF airway epithelial cells indicated that activating heterotrimeric G-proteins by

GTP-γ-S inhibited CFTR Cl^- currents [30]. These studies also suggested that inhibiting these G-proteins might activate mutant CFTR Cl^- currents in CF airway epithelial cells with a potential therapeutic value [30]. However, the present findings indicate that in this absorptive epithelium, G-proteins mediate activation, not inhibition, of CFTR G_{Cl} not only in normal ducts (Fig. 1), but also to some extent in a duct from a CF patient (Fig. 7). The apparent discrepancy between cultured airway epithelial cells and native sweat duct epithelial cells suggest that G-protein regulation of CFTR may be tissue specific or simply that cultured airway epithelial cells and native sweat duct cells may reflect very different histories and functional demands.

Conclusions

We have shown for the first time that trimeric G-proteins are expressed in both apical and basolateral membranes of the native human sweat duct and that CFTR in the apical membrane can be activated by activating a heterotrimeric G-protein in the presence of ATP. A member of the Gαs family of G-proteins seems most likely to regulate CFTR in microdomains of the apical membrane. G-protein activation seems to be at least partially effective in activating a poorly expressed mutant form of CFTR.

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