cAMP-independent phosphorylation activation of CFTR by G proteins in native human sweat duct

M. M. REDDY AND P. M. QUINTON
Department of Pediatrics, School of Medicine, University of California, San Diego, La Jolla, California 92093-0831

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Reddy, M. M., and P. M. Quinton. cAMP-independent phosphorylation activation of CFTR by G proteins in native human sweat duct. Am J Physiol Cell Physiol 280: C604–C613, 2001.—It is generally believed that cAMP-dependent phosphorylation is the principle mechanism for activating cystic fibrosis transmembrane conductance regulator (CFTR) Cl channel. However, we showed that activating G proteins in the sweat duct stimulated CFTR Cl conductance (G_{Cl}) in the presence of ATP alone without cAMP. The objective of this study was to test whether the G protein stimulation of CFTR G_{Cl} is independent of protein kinase A. We activated G proteins and monitored CFTR G_{Cl} in basolaterally permeabilized sweat duct. Activating G proteins with guanosine 5’-O-(3-thiotriphosphate) (10–100 μM) stimulated CFTR G_{Cl} in the presence of 5 mM ATP alone without cAMP. G protein activation of CFTR G_{Cl} required Mg^{2+} and ATP hydrolysis (5’-adenylylimidodiphosphate could not substitute for ATP). G protein activation of CFTR G_{Cl} was I) inhibition by the kinase inhibitor staurosporine (1 μM), indicating that the activation process requires phosphorylation; 2) insensitive to the adenylate cyclase (AC) inhibitors 2’,5’-dideoxyadenosine (1 mM) and SQ-22536 (100 μM); and 3) independent of Ca^{2+}, suggesting that Ca^{2+}-dependent protein kinase C and Ca^{2+}/calmodulin-dependent kinase(s) are not involved in the activation process. Activating AC with 10^{-6} M forskolin plus 10^{-6} M IBMX (in the presence of 5 mM ATP) did not activate CFTR, indicating that cAMP cannot accumulate sufficiently to activate CFTR in permeabilized cells. We concluded that heterotrimeric G proteins activate CFTR G_{Cl} endogenously via a cAMP-independent pathway in this native absorptive epithelium.

heterotrimeric G protein; cystic fibrosis; SQ-22536; dideoxyadenosine; electrolyte transport; absorption; fluid transport regulation

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tempts to deactivate CFTR by pharmacologically inhibiting cAMP production have not been successful (19). These results suggest that the predominant mechanism for activating CFTR in this absorptive epithelium might involve a G protein-activated mechanism that is independent of a cAMP cascade.

G proteins are a family of membrane-bound proteins that exist in both monomeric and heterotrimeric forms (2, 25, 29). The general scheme of signal transduction by heterotrimeric G proteins involves $\alpha_\gamma$ heteromers. When a receptor linked to G proteins is activated, the GTP binds to the $\alpha$-subunit of the G protein complex and liberates it from the $\beta_\gamma$ complex. Both $\alpha$-GTP complex and $\beta_\gamma$ complex are known to regulate cellular events (3, 8, 10). G proteins may regulate ion channels by different mechanisms including 1) regulation of AC/cAMP/PKA cascade-dependent phosphorylation; 2) regulating protein kinase C (PKC)-dependent phosphorylation through inositol phosphate metabolites and diacylglycerol, for example; and 3) direct interaction with channel proteins (2, 3, 8, 10).

The objective of this investigation was to determine whether AC and PKA phosphorylation mediate the G protein regulation of CFTR in NaCl absorption endogenously. We found that phosphorylation is involved in the G protein-induced activation of CFTR in the apical membranes of sweat duct but that, unexpectedly, such activation of CFTR appears to be independent of the AC/cAMP cascade in this native tissue.

**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 solution without medical history who gave informed consent. Individ-

**Select Permeabilization of the Basolateral Membrane**

The basolateral membrane of the sweat duct was selectively permeabilized with a pore-forming agent ($\alpha$-toxin; 1,000 U/ml derived from *Staphylococcus aureus*) in cytoplasmic Ringer solution containing 140 mM K-glucronate and 5 mM ATP applied to the basolateral surface of the microperfused sweat duct for 15–30 min. As described earlier, $\alpha$-toxin effectively removes the basolateral membrane as a barrier to cAMP and ATP without affecting the functional integrity of the apical membrane. This preparation allows free manipulation of intracellular cAMP, ATP, and GTP so that the properties of the regulation of CFTR $G_{Cl}$ in the apical membrane can be studied in relative isolation from their endog-

**Electrical Measurements**

**Electrical setup.** After the lumen of the sweat duct had been cannulated with a double-lumen cannula made from 0–
glass, a constant current pulse of 50–100 nA was injected for a duration of 0.5 s through one barrel of the cannulating pipette containing NaCl Ringer 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 by using one channel of a WPI-700 dual electrometer referenced to the contraluminal bath. Transepithelial conductance ($G_t$) was calculated from the cable equation as described earlier (9, 17) by using the measured amplitude of the $V_t$ deflections in response to the transepithelial constant current pulse.

**Apical Cl$^-$ conductance.** Cl$^-$ diffusion potentials ($V_{Cl}$) and $G_{Cl}$ were monitored as indicators of the level of activation of $G_{Cl}$. Treatment with $\alpha$-toxin to permeabilize the basolateral membrane simplified the epithelium to a single (apical) mem-

**Solutions**

The luminal perfusion R solutions contained (in mM) 150 NaCl, 5 K$^+$, 3.5 PO$_4^{3-}$, 1.2 MgSO$_4$, 1 Ca$^{2+}$, and 0.01 amiloride, pH 7.4. Cl$^-$-free luminal Ringer solution was prepared by complete substitution of Cl$^-$ with the impermeant anion gluconate. The cytoplasmic bath solution contained (in mM) 145 K$^+$, 140 gluconate, 3.5 PO$_4^{3-}$, and 1.2 MgSO$_4$, as well as 260 $\mu$M Ca$^{2+}$ buffered with 2.0 mM EGTA (Sigma) to 80 mM free Ca$^{2+}$, pH 6.8. Nominally Mg$^{2+}$-free cytoplasmic bath solution with 5 mM EDTA was used to prepare Mg$^{2+}$-free solution. Nominally Ca$^{2+}$-free cytoplasmic bath solution was prepared by adding 2 mM EGTA to Ca$^{2+}$-free cytoplasmic bath solution. ATP (5 mM), adenosine 5'-O-(3-thiotriphosphate) (ATP$\gamma$S; 5 mM), cAMP (0.1 mM), GTP$\gamma$S (0.1 mM), 5'-adenylylimidodiphosphate (AMP-PNP; 5 mM), AICl$_3$ (0.1 mM), KF (5 mM), 2,5'-dideoxyadenosine (DDA; 0.05–1 mM), and SQ-22536 (0.1) were added to the cytoplasmic bath as needed.

**Data Analysis**

$V_{Cl}$ and $G_{Cl}$ in bar graphs represent peak values that were stable for at least 2 min within ±2 mV. Data are presented as means ± SE ($n$ = 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 to be significantly different. Data presented as representative examples are taken from similar experiments repeated at least three times.

**RESULTS**

**Effect of GTP$\gamma$S**

After basolateral $\alpha$-toxin permeabilization of the sweat duct cytoplasmic nucleotides, responsible for activating CFTR, leak out of the cell and CFTR deactivates spontaneously as indicated by a virtually complete lack of $G_{Cl}$ and $V_{Cl}$ across the apical membrane. Reactivation of CFTR requires the presence of both 0.1
mM cAMP and 5 mM ATP in the cytoplasmic bath. Removing cAMP deactivates CFTR even in the presence of ATP, showing endogenous dephosphorylation of CFTR. However, application of 10–100 μM GTPγS was applied to the cytoplasmic side in the complete absence of ATP. Excess 100 μM GTPγS in the bath was washed out. Under these conditions, application of 5 mM ATP activated CFTR conductance (GCl) independent of cAMP. This effect of GTPγS was not mimicked by ATPγS, indicating that phosphatase-resistant “irreversible” phosphorylation of CFTR by GTPγS was not responsible for the observed results. These results indicate that CFTR GCl is regulated by G proteins in the native sweat duct.

Fig. 1. The effect of adenosine 5′-O-(3-thiotriphosphate) (ATPγS) and guanosine 5′-O-(3-thiotriphosphate) (GTPγS) on ATP activation of cystic fibrosis transmembrane conductance regulator (CFTR). A: GTPγS was applied to the cytoplasmic side in the complete absence of ATP. Excess 100 μM GTPγS in the bath was washed out. Under these conditions, application of 5 mM ATP activated CFTR conductance (GCl) independent of cAMP. This effect of GTPγS was not mimicked by ATPγS, indicating that phosphatase-resistant “irreversible” phosphorylation of CFTR by GTPγS was not responsible for the observed results. These results indicate that CFTR GCl is regulated by G proteins in the native sweat duct. B: summary of data collected from experiments similar to that shown in A. Notice that after G protein activation, ATP alone stimulated CFTR GCl comparable to activation with cAMP + ATP before G protein activation (n = 7, P < 0.001) as indicated by similar increases in Cl− diffusion potentials (VCl) and Cl− conductance (CFTR GCl).

Fig. 2. G protein activation of CFTR GCl in the presence of ATP is Mg2+ dependent. A: this experiment tested whether ATP activation of CFTR requires Mg2+. Notice that G protein can be activated by GTPγS in the complete absence of Mg2+ as indicated by subsequent ATP activation of CFTR in the presence of Mg2+. B: after the G proteins are activated, ATP activation of CFTR critically requires Mg2+ because we could not activate CFTR GCl in the absence of Mg2+ in the cytoplasmic bath. These results indicate that ATP hydrolysis and a phosphorylation process probably are necessary to activate CFTR GCl. C: summary of data collected from experiments similar to that in B, showing a significant inhibition of G protein/ATP-induced CFTR GCl activity by Mg2+ removal from the cytoplasmic bath (n = 3, P < 0.02).
Effect of Inhibiting Phosphorylation

Removing Mg\(^{2+}\) from the cytoplasmic bath significantly inhibited ATP activation of CFTR after GTP\(_{\gamma}\)S was applied to the duct. The magnitude of ATP-induced CFTR \(G_{C_{1}}\) and \(V_{C_{1}}\), respectively, was 56.4 ± 14.3 mS/cm\(^2\) and 51.7 ± 16.4 mV in the presence of Mg\(^{2+}\) but only 6.8 ± 4.4 mS/cm\(^2\) and 3.8 ± 4.1 mV in the nominal absence of Mg\(^{2+}\) (\(n = 3, P < 0.02\); Fig. 2). However, Mg\(^{2+}\) was not required for GTP\(_{\gamma}\)S activation of G proteins because application of GTP\(_{\gamma}\)S in the complete absence of Mg\(^{2+}\) resulted in sustained activation of G proteins. This effect was shown by the subsequent, prompt activation of CFTR \(G_{C_{1}}\) when ATP and Mg\(^{2+}\) were reintroduced without GTP\(_{\gamma}\)S (Fig. 2). However, after G proteins were similarly preactivated, the nonhydrolyzable ATP analog AMP-PNP (5 mM) did not activate CFTR (Fig. 3). Likewise, ATP failed to activate CFTR \(G_{C_{1}}\) after preactivating G proteins when endogenous kinases were inhibited by the nonselective kinase inhibitor staurosporine (10\(^{-6}\) M) (Fig. 4).

Effect of cAMP-Elevating Agents

We tested the effect of cAMP-elevating agents on both the intact unpermeabilized and the \(\alpha\)-toxin-permeabilized ducts. In the intact unpermeabilized ducts, CFTR \(G_{C_{1}}\) was monitored as the change in \(G_{t}\) (indicated by the magnitude of voltage deflections associated with transepithelial constant current pulses) and \(V_{t}\) (which included either 1) spontaneous potentials in 150 mM NaCl bilaterally or 2) diffusion potentials generated by 150 mM Na-gluconate in the lumen and 150 mM NaCl in the contraluminal bath). Application of forskolin (to activate AC) and IBMX (to inhibit phosphodiesterase) increased CFTR \(G_{C_{1}}\) in cAMP-responsive native sweat ducts,\(^2\) as indicated by an increase in \(G_{t}\) and corresponding changes in \(V_{t}\) (Fig. 5). In contrast, application of a cocktail of the cAMP-elevating agents forskolin and IBMX to the cytoplasm in the presence of ATP had no detectable effect on CFTR in basolaterally permeabilized sweat duct (Fig. 5). These results may indicate that small solutes such as cAMP cannot accumulate sufficiently to activate CFTR in permeabilized duct. Moreover, after permeabilization, the apical membrane conductance dramatically decreased, consistent with the loss of cytosolic CFTR-activating substances. Exogenous addition of cAMP or GTP\(_{\gamma}\)S in the presence of ATP rapidly restored CFTR \(G_{C_{1}}\). These results show that permeabilizing the basolateral membrane with \(\alpha\)-toxin probably depletes the cytoplasm of small molecules such as cAMP, cGMP, ATP, and GTP.

\(^2\)Not all intact ducts respond to cAMP-mediated agonist because CFTR is usually spontaneously activated in the duct, presumably to its maximal activated state.

Fig. 3. ATP hydrolysis is required for activating CFTR after G protein activation. This experiment tested whether ATP hydrolysis is required for activating CFTR \(G_{C_{1}}\) after G proteins are activated. In this experiment, 100 \(\mu\)M GTP\(_{\gamma}\)S was applied to the cytoplasmic bath to activate G proteins before application of the nonhydrolyzable ATP analog 5′-adenylylimidodiphosphate (AMP-PNP) or physiological ATP. Notice that AMP-PNP had little effect, whereas ATP significantly activated CFTR \(G_{C_{1}}\), indicating that ATP hydrolysis is required for G protein-mediated CFTR \(G_{C_{1}}\) activation.
Effect of Inhibiting AC

Inhibiting AC with 1 mM DDA (a membrane-permeable inhibitor of AC) in the bath did not inhibit CFTR $G_{Cl}$ in nonpermeabilized intact duct as indicated by the lack of effect of DDA on transepithelial $G_{Cl}$ and $V_{Cl}$ (Fig. 6). Application of either DDA (50 μM or 1 mM) or SQ-22536 (another AC inhibitor; 100 μM) in the cytoplasmic bath of basolaterally permeabilized duct also had little effect on G protein-induced activation of CFTR in the presence of ATP (Fig. 7). After G protein-induced activation, ATP increased CFTR $G_{Cl}$ and $V_{Cl}$, respectively, by 36.8 ± 6.7 mS/cm² and 47.1 ± 11.3 mV in the presence of DDA (1 mM) and by 38.9 ± 7.3 mS/cm² and 53.0 ± 10.5 mV in the absence of DDA ($n = 7$). These results indicate that AC is not requisite to activate CFTR $G_{Cl}$.

Effect of Ca²⁺

We tested whether the G protein effector might require Ca²⁺ by removing Ca²⁺ from the cytoplasmic bath. Nominally Ca²⁺-free EGTA-buffered Ringer so-

![Image](http://apcell.physiology.org)
Solution in the cytoplasm had little effect on either GTPγS or AlF4−-mediated ATP activation of CFTR (Fig. 8).3 After G protein activation, ATP increased CFTR GCl and VCl, respectively, by 29.3 ± 6.3 mS/cm² and 48.7 ± 6.3 mV in the presence of Ca²⁺ (80 nM) and by 30.9 ± 2.8 mS/cm² and 42.0 ± 5.4 mV in the complete absence of Ca²⁺ (n = 4). Thus it seems unlikely that G protein activation of CFTR requires a Ca²⁺-dependent pathway.

Lack of Synergistic Effect of GTPγS With cAMP

The effect of cAMP- and G protein-induced activation on the magnitude of CFTR GCl was comparable. Application of cAMP after G proteins were activated with GTPγS did not increase the ATP activation of CFTR GCl (Fig. 9).

DISCUSSION

The apical membrane of the reabsorptive sweat duct expresses a number of heterotrimeric G proteins, including Gα, Gα, Gα, Gα, and Gβ (unpublished immunocytochemical observations). It is well known that these heterotrimeric G proteins control the activity levels of a number of protein kinases, including those responsible for phosphorylation activation of CFTR such as PKA and PKC (2, 3, 7, 10). However, it is also known that regulation of a number of G protein-mediated ion channels involves direct interaction between the channel protein and the G protein (2, 3, 8, 10). Therefore, we also examined whether the activation of CFTR GCl by the apical G proteins involves phosphorylation or a direct interaction between CFTR and the G protein. Because kinase phosphorylation is involved in the G

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3 AlF4− is commonly used to activate heterotrimeric G proteins as opposed to monomeric forms.
protein-mediated activation of CFTR, we investigated the possible role of cAMP/PKA cascade in the G protein-mediated activation of CFTR $G_{Ci}$ by ATP alone (in the absence of exogenous cAMP) in the permeabilized duct.

**G Protein-Induced Activation of CFTR Requires Phosphorylation**

Kinase phosphorylation critically requires Mg$^{2+}$ (20). Removing Mg$^{2+}$ from the cytoplasmic bath before application of ATP prevented subsequent activation of CFTR by ATP (Fig. 2). However, Mg$^{2+}$ also plays a critical role in GTP$\gamma$S binding to the G proteins and in ATP hydrolysis (8, 20). The Ga-GTP$\gamma$S-Mg$^{2+}$ complex is extremely stable, favoring $\beta\gamma$-subunit dissociation and activation of the G protein interaction with the target proteins (8). Therefore, we tested whether the lack of effect of ATP on CFTR in the absence of Mg$^{2+}$ is due to the failure of GTP$\gamma$S binding to the G protein. We exposed the apical membranes to GTP$\gamma$S for ~1 min in the complete absence of Mg$^{2+}$ and then washed out GTP$\gamma$S with Mg$^{2+}$-free solution. Subsequent addition of ATP in the presence of Mg$^{2+}$ activated CFTR $G_{Ci}$. These results are surprising in light of previous reports that removing Mg$^{2+}$ destabilizes Ga-GTP$\gamma$S, increases the rate of dissociation of GTP$\gamma$S from Ga, and increases the association of $\alpha$-subunits with $\beta\gamma$-subunits, thereby deactivating the G protein (8). These results suggest that 1) the apical G proteins may be unique in not requiring Mg$^{2+}$ for GTP$\gamma$S binding to the Ga or 2) other divalent cations such as Ca$^{2+}$ may replace Mg$^{2+}$ in facilitating GTP$\gamma$S binding to Ga. Therefore, the failure of CFTR $G_{Ci}$ to activate with ATP in the absence of Mg$^{2+}$ is most likely due to a need for higher Mg$^{2+}$ levels for phosphorylation or hydrolysis of CFTR than for GTP$\gamma$S binding to the G protein.

We tested this possibility further by studying the effect of the nonhydrolyzable ATP analog AMP-PNP on CFTR $G_{Ci}$ after activating G proteins with GTP$\gamma$S (21). As shown in Fig. 3, only ATP, not AMP-PNP, activated CFTR $G_{Ci}$, confirming that ATP hydrolysis is required at one or more steps in the G protein cascade that activates CFTR. Because ATP hydrolysis is involved at two different kinetic steps, one requiring and the other independent of phosphorylation (20, 21), we tested whether ATP hydrolysis reflects the phosphorylation process. Although staurosorine is nonspecific, Fig. 4 shows that this inhibitor apparently prevented ATP activation of CFTR $G_{Ci}$, presumably by blocking endogenous kinase activity. These results indicate that a kinase-dependent phosphorylation step is required in G protein-induced activation of CFTR.

**GTP$\gamma$S Does Not Irreversibly Phosphorylate CFTR**

CFTR $G_{Ci}$ clearly can be activated by PKA, which is ATP and Mg$^{2+}$ dependent (20, 21). Previous studies also showed that CFTR can be irreversibly phosphorylated by using ATP$\gamma$S as substrate for PKA phosphorylation (20, 21). Under these conditions, CFTR $G_{Ci}$ remained activated as long as ATP was present because the thionophosphorylated CFTR cannot be dephosphorylated (20, 21). Because application of GTP$\gamma$S also activated CFTR $G_{Ci}$ as long as ATP was present (Fig. 1), it is possible that GTP$\gamma$S might thionophosphorylate CFTR by either a constitutively active kinase or a G protein-activated kinase using GTP$\gamma$S as substrate. The possibility that an endogenously active kinase is responsible for CFTR phosphorylation is ruled out by the facts that 1) an equimolar concentration of ATP$\gamma$S in the absence of cAMP failed to thionophosphorylate CFTR (CFTR was not subsequently activated by ATP alone; Fig. 1) and 2) previous studies showed that once CFTR was irreversibly thionophosphorylated, CFTR remained independent of Mg$^{2+}$ (21). However, normal ATP phosphorylation activation of CFTR $G_{Ci}$ critically depended on the presence of Mg$^{2+}$ in the cytoplasmic bath. Removing Mg$^{2+}$ after prior activation of CFTR $G_{Ci}$ in the presence of Mg$^{2+}$ and ATP deactivated CFTR $G_{Ci}$, possibly because endogenous phosphatase dephosphorylation overtook the Mg$^{2+}$-dependent phosphorylation process (Figs. 2 and 3) or because CFTR gating is Mg$^{2+}$ dependent at some level. Also, in the presence of staurosorine, ATP failed to activate CFTR $G_{Ci}$ previously exposed to GTP$\gamma$S. If CFTR had been irreversibly phosphorylated by GTP$\gamma$S, inhibiting the kinase by staurosorine would not have prevented ATP activation of CFTR $G_{Ci}$, as shown in Fig. 4. Together, these results argue that G protein-induced activation in the presence of GTP$\gamma$S and ATP does not cause irreversible thionophosphorylation of CFTR. In contrast, they suggest that activating the G proteins activates an as yet unknown kinase phosphorylation of CFTR.

**PKA Phosphorylation Is Not Required to Activate CFTR**

If G protein-mediated activation of CFTR requires phosphorylation but is not thionophosphorylated by
GTPγS, as concluded above, then, we asked, does PKA mediate the phosphorylation activation? The apical membrane of this absorptive epithelium shows immunocytochemical labeling consistent with the presence of Gαs (unpublished observation). Furthermore, Gαs commonly stimulates AC to increase intracellular cAMP levels in a number of tissues (2, 3, 10). It is therefore tempting to assume that an apical Gαs activates an AC/cAMP/PKA-dependent phosphorylation activation of CFTR GCl. However, the following studies indicate that G protein-induced activation of CFTR does not involve the AC/cAMP regulatory cascade.

No cAMP accumulation in permeabilized duct cells. The intact nonpermeabilized sweat duct has significant K+ and Cl− conductances in the basolateral membrane and Na+ and Cl− conductances in the apical membrane (17–20). Complete substitution of NaCl in the contraluminal bath with equimolar K-gluconate significantly depolarizes the basolateral membrane and transepithelial potentials (20, 21). Permeabilizing the basolateral membrane with α-toxin removes the basolateral membrane as a functional barrier so that intracellular cAMP cannot accumulate. After α-toxin, first, K+ and Cl− diffusion potentials across the basolateral membrane were abolished (Vt of about +11 mV reflects the junction potential), and the K+ conductance inhibitor (Ba2+) or Na+-K+-pump inhibitor (ouabain) had no effect on basolateral membrane potential after permeabilization (20); second, isoproteenol (β-adrenergic agonist) variably induced activation of CFTR GCl (19) possibly by increasing intracellular cAMP levels via a G protein-coupled mechanism (7, 31) but did not have an effect on the Cl− conductance of permeabilized ducts (results not shown). More specifically, the AC activator forskolin and the phosphodiesterase inhibitor IBMX together activated CFTR GCl in some nonpermeabilized ducts but never in α-toxin-permeabilized ducts (Fig. 5). These results suggest that no newly synthesized cAMP does not accumulate sufficiently inside the cell to activate PKA phosphorylation of CFTR (Fig. 5). This conclusion is further corroborated by the fact that during α-toxin permeabilization, the apical CFTR GCl becomes almost completely deactivated but can be reactivated quickly by the addition of exogenous cAMP and ATP to the cytoplasmic bath perfusate (Fig. 1) (20).

No effect of inhibiting AC on G protein-induced activation of CFTR GCl. CFTR GCl is maximally activated in a majority of the isolated microperfused sweat ducts (19). One possible explanation for such persistent activation of CFTR GCl could be that intracellular cAMP levels are elevated because of continuous G protein stimulation of AC. If this were the case, CFTR GCl should be deactivated by inhibiting AC. There are about 10 different isoforms of AC in mammalian tissues (31). DDA and SQ-22536 inhibit all known forms of AC and block cAMP production. Thus we tested the effect of AC inhibitors on the Cl− conductance of intact nonpermeabilized ducts. CFTR GCl remained high and unaffected by DDA (even at 1 mM), suggesting that intracellular cAMP is not responsible for the constitutive, persistent activation of CFTR in the native sweat duct (Fig. 6). We also tested the effect of DDA and SQ-22536 in the cytoplasmic bath on GTPγS/ATP activation of CFTR GCl in the permeabilized duct to be certain that the inhibitors diffused into the cell and that the microdomains of AC/PKA did not escape inhibition. Figure 7 shows that these inhibitors did not prevent G protein-mediated activation of CFTR GCl. These results strongly indicate that G protein-mediated signal transduction associated with CFTR GCl activation does not involve an AC/cAMP cascade in this salt-absorbing epithelium.

Phosphorylation is Ca2+ Independent

Do G proteins also effect signal transduction through phospholipase C and PKC (12). Because Ca2+ plays a significant role in PKC- and calmodulin-dependent kinases, we tested the effect of removing Ca2+ on both cAMP- and GTPγS-mediated activation of CFTR GCl. Figure 8 shows that removing Ca2+ did not have an effect on the magnitude of G protein-mediated ATP activation of CFTR GCl, indicating the finding that Ca2+-dependent pathways do not play a direct role in the G protein-activated induction of CFTR.
sweat duct. Further investigation is needed to determine which of the numerous protein kinase(s) is specifically involved in G protein-induced phosphorylation activation of CFTR in the sweat duct.

**Why Are CFTR Cl\(^-\) Channels Constitutively Open in the Duct?**

CFTR G\(_{Cl}\) is constitutively activated in sweat duct cells and under some culture conditions in Calu-3 cells derived from the airways, as well (11, 19). There are at least three distinct possibilities that could explain this phenomenon. First, it is possible that the endogenous levels of cAMP are consistently elevated so that PKA keeps CFTR phosphorylated. However, this does not seem to be a viable explanation because we could not deactivate CFTR after inhibiting cAMP production, conditions that should lead to dephosphorylation. Overnight incubation of ducts in a cocktail containing inhibitors of cAMP production, including propranolol (to block \(\beta\)-adrenergic receptor) and indomethacin (to block cyclooxygenase and prostaglandin synthesis), did not inhibit CFTR G\(_{Cl}\) (15, 19). In addition, inhibiting AC in intact nonpermeabilized sweat ducts with DDA or SQ-22536 did not inhibit the constitutively open CFTR G\(_{Cl}\) (Fig. 6), suggesting that elevated cAMP levels are less likely to be the cause of constitutively active CFTR G\(_{Cl}\). Second, very low endogenous phosphodiesterase or phosphatase activities in the intact duct cells may allow CFTR to remain activated. However, we know that CFTR G\(_{Cl}\) is rapidly dephosphorylated by active endogenous phosphatases in permeabilized cells (22) so that low phosphodiesterase/phosphatase activities seem not to explain constitutive CFTR G\(_{Cl}\) activation. Third, chronically activated receptors may constitutively stimulate apical G proteins to activate CFTR as long as appropriate levels of ATP exist in the cell. The observation that once GTP\(_\gamma\)S was bound to the G proteins, CFTR remained activated in the presence of ATP alone is consistent with, but not proof of, this notion.

**What Triggers the G Protein-Mediated Activation of CFTR G\(_{Cl}\)?**

Unpublished results involving the use of immunocytochemical labeling techniques revealed the presence of G\(_{\alpha}\), G\(_{\beta}\), and G\(_{\delta}\) in the apical membrane. These observations suggested that G proteins in the apical membrane control CFTR in the sweat duct. As discussed above, if activation of the apical G proteins is, in fact, responsible for constitutively opening CFTR Cl\(^-\) channels in the apical membrane, we must ask, what sustains stimulation of the G proteins in the intact duct? Luminal perfusate (NaCl-containing Ringer solution) is devoid of neurohumoral agents that might otherwise stimulate G proteins. Early reports indicated that changes in the ionic environment might regulate G protein activation. Changes in the cytosolic Cl\(^-\) concentration were reported to alter G protein regulation of epithelial Na\(^+\) channel function in salivary duct epithelial cell (4). Changes in Cl\(^-\) concentrations appeared to inhibit GTPase activity [hence, to activate G protein (8)]. We therefore tested the effect of increasing cytosolic Cl\(^-\) concentration from 0 to 140 mM on G protein activation of CFTR. We found that cytosolic Cl\(^-\) had little effect on CFTR G\(_{Cl}\) activation by GTP\(_\gamma\)S in the presence of ATP (results not shown). It is known that luminal [NaCl] changes from isotonic to <15 mM as a function of secretory rate (1, 24). We asked whether changes in [Na\(^+\)] have an effect on G protein activation of CFTR. We found that removing Na\(^+\) (by substituting with K\(^+\)) did not prevent GTP\(_\gamma\)S activation of CFTR G\(_{Cl}\) (results not shown). Further studies are required to determine the mechanisms that activate apical G proteins and CFTR.

**Implications for Cystic Fibrosis**

CFTR G\(_{Cl}\) is significantly reduced or almost completely absent in most CF-affected epithelium (13, 14, 30). Until now, it has been widely believed that cAMP-dependent phosphorylation of CFTR is the predominant physiological mechanism for activating CFTR G\(_{Cl}\) in a number of epithelial cells in airways, pancreas, intestine, and sweat glands (13, 14, 30). However, our results here suggest that the G protein-induced signal transduction leading to the activation of CFTR may not involve AC/cAMP cascade. Earlier studies on cultured airway epithelial cells indicated that activating the G proteins inhibits CFTR Cl\(^-\) channels (29). However, it is unclear whether such inhibition is a generalized phenomenon applicable to all the transporting cells (i.e., secretory as well as absorptive cells) within the airways or whether the cells performing absorptive function in the airways exhibit G protein-induced activation of CFTR similar to that in sweat duct cells. Potential therapeutic strategies aimed at modulating the G protein regulation of CFTR within the airways must take into account possible differential effects of G proteins on CFTR as a dependent function of vectorial transport (absorption vs. secretion) in different cell types.

**Conclusion**

G proteins activate CFTR G\(_{Cl}\) in the native sweat duct. Kinase phosphorylation is involved in the G protein-mediated CFTR G\(_{Cl}\) activation, but the AC/cAMP cascade may not play a direct role in this regulatory process.

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