G-protein Regulation of Outwardly Rectified Epithelial Chloride Channels Incorporated into Planar Bilayer Membranes*

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Experiments were designed to test if immunopurified outwardly rectified chloride channels (ORCCs) and the cystic fibrosis transmembrane conductance regulator (CFTR) incorporated into planar lipid bilayers are regulated by G-proteins, pertussis toxin (PTX) (100 ng/ml) + NAD (1 mm) + ATP (1 mm) treatment of ORCC and CFTR in bilayers resulted in a 2-fold increase in single channel open probability (Po) of ORCC but not of CFTR. Neither PTX, NAD, nor ATP alone affected the biophysical properties of either channel. Further, PTX conferred a linearity to the ORCC current-voltage curve, with a slope conductance of 80 ± 3 picosiemens (pS) in the ± 100 mV range of holding potentials. PKA-mediated phosphorylation of these PTX + NAD-treated channels further increased the P0 of the linear 80-pS channels from 0.66 ± 0.05 to >0.9, and revealed the presence of a small (16 ± 2 pS) linear channel in the membrane. PTX treatment of a CFTR-immunodepleted protein preparation incorporated into bilayer membranes resulted in a similar increase in the P0 of the larger conductance channel and restored PKA-sensitivity that was lost after CFTR immunodepletion. The addition of guanosine 5'3'-O-(thio)triphosphate (100 μM) to the cytoplasmic bathing solutions decreased the activity of the ORCC and increased its rectification at both negative and positive voltages. ADP-ribosylation of immunopurified material revealed the presence of a 41-kDa protein. These results demonstrate copurification of a channel-associated G-protein that is involved in the regulation of ORCC function.

Cystic fibrosis (CF) is an autosomal recessive disease that is common in North America. A characteristic of the disease is impaired Cl- transport across several tissues including those of the airway epithelia. The gene responsible for impaired Cl- transport encodes the cystic fibrosis transmembrane conductance regulator (CFTR), a protein that acts as a small linear Cl- channel at the plasma membrane. A second Cl- channel in the apical membrane of affected tissue (the so-called outwardly rectified chloride channel or ORCC) is also affected in CF such that it cannot be activated by PKA and ATP in cells with the CF phenotype. The presence of a functional CFTR in the membrane is required for the PKA/ATP activation of the ORCC (1–3). The biophysical properties of ORCC and CFTR Cl- channels are distinct and well established. ORCCs have a nonlinear current-voltage (I/V) relationship with a 20–40-pS single-channel conductance at hyperpolarizing voltages and a 60–80-pS conductance at depolarizing voltages (3–8). ORCCs are blocked by a wide variety of molecules including DIDS and the calcium channel blockers (9) and have a halide permeability sequence of I- > Cl- > Br- > I-. ORCCs can be activated by PKA and protein kinase C (10–13). Conversely, the CFTR Cl- channel has a linear I/V relationship with an 8–16-pS single-channel conductance. Activity channel can be blocked by diphenylamine-2-carboxylic acid (DPC) and glibenclamide (13), but not by DIDS. The halide permeability sequence for CFTR is Br- > Cl- > I-.1

Heterotrimeric G-protein binding proteins (G-proteins) regulate ion channels in a variety of tissues including respiratory epithelia (16). Regulation can be direct or indirect through a cytoplasmic pathway involving second messengers and protein kinases (16, 17). Heterotrimeric G-proteins can also regulate intracellular vesicle trafficking (18). Both regulatory mechanisms (direct regulation and regulation of endo- and exocytosis) have been implicated in cAMP-dependent Cl- secretion in normal and CF epithelia (17, 18). It was shown in whole-cell patch clamp studies (18) that pertussis toxin (PTX), which uncouples heterotrimeric G, proteins from their receptors, increases Cl- transport and restores cAMP-activated Cl- currents in airway epithelial cells isolated from CF patients. Additional studies suggested that the heterotrimeric G-protein Gai-2 regulates CFTR Cl- conductance in human airway epithelial cells by modulating vesicle trafficking and the delivery of CFTR Cl- channels from an intracellular vesicular pool to the plasma membrane (18). The same authors also demonstrated that the only pertussis toxin-sensitive G-protein expressed in human airway cells was Gai-2 (18). As the apical membrane of airway epithelial cells contains two cAMP-activated Cl- channels (ORCC and CFTR), the possibility exists that both of them are regulated by G-proteins. Gai-2 inhibits CFTR function solely by preventing trafficking of the protein to the apical membrane. However, Gai-2 may regulate ORCCs by a direct mechanism independent of second messenger involvement (17, 18).

We recently reported the simultaneous isolation and functional reconstitution of an ORCC and CFTR from apical membrane vesicles of bovine tracheal epithelial cells (3). Isolated channels were functionally preserved and exhibited similar regulatory features as native channels recorded in patch clamp studies from airway epithelia. In light of these observations, the goals of this study were to determine whether a PTX-sensitive G-protein copurified with these Cl- channels and to...
examine the regulatory relationship between the isolated channels and the copurified G-protein.

**EXPERIMENTAL PROCEDURES**

Materials

$[^{32}P]NAD$ was obtained from NEN-DuPont. A hydrazide-derivatized Acti-Disk was obtained from FMC Corporation (Pine Brook, NJ). Peroxide-free Triton X-100, and SM2-Biobeads were from Bio-Rad. Phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). All other reagents were of analytical grade and were purchased from Sigma, Bio-Rad, or Fisher (Pittsburgh, PA).

Methods

**Tracheal Apical Membrane Preparation**—Apical membrane vesicles were prepared by differential centrifugation using a procedure first described by Langridge-Smith et al. (20), modified as described previously (21). Aliquots of vesicles (average protein concentration, 5 mg/ml) were stored in liquid nitrogen until use. Extraction of peripheral proteins from native membrane vesicles was achieved by incubation of vesicles for 30 min in KCl buffer (100 mM KCl, 5 mM Tris/Hepes, and 0.5 mM MgCl$_2$) titrated to pH 10.8 with 0.1 M NaOH. Alkaline-stripped vesicles were centrifuged at 35,000 $\times$ g for 35 min. These vesicles were isolated by centrifugation with NaCl/phosphate buffer before elution with 100 mM glycine, pH 3.

**Pertussis Toxin-induced ADP-ribosylation of immunopurified anion channel proteins from bovine tracheal epithelia.** Pertussis toxin induced ADP-ribosylation of solubilized protein from apical tracheal vesicles (lane 1) and proteins immunopurified with p38 antibodies before CFTR precipitation (lane 2) and after CFTR precipitation (lane 3). Specificity of ribosylation was confirmed by lack of 41-kDa ribosylated protein (lane 4) when immunopurified proteins were not added to the reaction mixture.

**Identification of a Pertussis-sensitive G Protein in Tracheal Membranes**—To determine if an inhibitory G-protein was present in our preparation, we examined the ability of PTX to ADP-ribosylate both solubilized tracheal membrane vesicles and the partially purified tracheal material that was used for incorporation into planar bilayers. ADP-ribosylation with PTX revealed the presence of a single band migrating at 40–41 kDa in solubilized tracheal membrane vesicles and in the protein material that contained both the ORCC and CFTR (Fig. 1: n = 4). A band at 40–41 kDa was also detected in the remaining material after CFTR precipitation (Fig. 1).

**RESULTS**

Identification of a Pertussis-sensitive G Protein in Tracheal Membranes—To determine if an inhibitory G-protein was present in our preparation, we examined the ability of PTX to ADP-ribosylate both solubilized tracheal membrane vesicles and the partially purified tracheal material that was used for incorporation into planar bilayers. ADP-ribosylation with PTX revealed the presence of a single band migrating at 40–41 kDa in solubilized tracheal membrane vesicles and in the protein material that contained both the ORCC and CFTR (Fig. 1: n = 4). A band at 40–41 kDa was also detected in the remaining material after CFTR precipitation (Fig. 1).

**Effects of GTP$_S$, PTX, and PKA on ORCC in Planar Lipid Bilayers**—In order to determine if G-proteins were involved in ORCC and CFTR channel regulation, we examined whether the addition of agents affecting the biochemical and functional state of G-proteins influenced the behavior of tracheal anion channels incorporated into planar lipid bilayers. In the absence of PKA + ATP (control), only a single, outwardly rectified channel could be identified (Figs. 2–4, top traces). GTP$_S$ (100 $\mu$M), a nonhydrolyzable analog of GTP, added to the cis or cytoplasmic side of the incorporated channel reduced the $P_o$ of this channel from 0.39 ± 0.06 to 0.18 ± 0.04 (n = 9). Subsequent addition of PKA (1.85 ng/ml) + ATP (100 $\mu$M) to the cis side of the bilayer induced the appearance of a second small conductance (16-pS) channel that was insensitive
PTX-sensitive G-proteins Regulate ORCC

Fig. 2. Effect of GTPγS and subsequent PKA phosphorylation on immunopurified ORCC and CFTR incorporated into planar lipid bilayers. Control channel recordings were performed at a holding potential of ±80 mV. The addition of 100 μM GTPγS from the cytoplasmic side decreased channel activity of the incorporated channel (from 0.39 to 0.18; n = 9). Consecutive addition of PKA (1.85 ng/ml) and ATP (100 μM) increased channel activity and induced the appearance of a small CFTR Cl⁻ channel on top of the opening and closing of the ORCC. The addition of 100 μM DIDS completely blocked ORCC, and only CFTR was recorded in this condition. 300 μM DPC blocked CFTR channel activity (bottom trace). The dashed line represents zero current. Records were filtered at 100 Hz.

to DIDS but was sensitive to DPC, a known blocker of CFTR (Fig. 2). When inhibitory anti-CFTR<sub>505-511</sub> antibodies were added subsequent to cis addition of PKA + ATP, the P<sub>o</sub> of the incorporated ORCC was 0.26 ± 0.05 (n = 9), not significantly different (<i>p > 0.01</i>) from that before PKA + ATP addition. The channel activity of CFTR (under conditions where the ORCC was inhibited by DIDS) was not appreciably altered in separate experiments by the addition of GTPγS (P<sub>o</sub> = 0.58 ± 0.07, n = 9, as opposed to P<sub>o</sub> = 0.63 ± 0.08, n = 9). We previously demonstrated that inhibitory anti-CFTR<sub>505-511</sub> antibodies inhibited CFTR channel activity and did not affect channel activity of the ORCC (19). Thus, these antibodies were used to facilitate the calculation of the P<sub>o</sub> of the ORCC by eliminating CFTR channel activity.

The addition of PTX (100 ng/ml), an agent known to inactivate G<sub>i</sub> proteins, together with NAD (1 mM) and ATP (1 mM), also significantly increased the P<sub>o</sub> of the ORCC from 0.38 ± 0.03 to 0.66 ± 0.05 (n = 11, Fig. 3). Furthermore, PTX conferred a linearity to the ORCC current-voltage curve, which had a slope conductance of 80 ± 3 pS at a holding potential of ±100 mV (see Fig. 5). As with GTPγS, PKA-mediated phosphorylation of these PTX + NAD-treated channels revealed the presence of a small (16 ± 2 pS), linear channel in the bilayer membrane (Fig. 3). Inhibition of the ORCC by DIDS afforded us the opportunity to compare the activity of phosphorylated CFTR following PTX treatment. There was no significant difference in the channel P<sub>o</sub> of CFTR for either the GTPγS-treated channel (P<sub>o</sub> = 0.63 ± 0.08 (n = 9); Fig. 2) or the PTX-treated channel (P<sub>o</sub> = 0.58 ± 0.05 (n = 9); Fig. 4).

To further explore the regulatory relationship between G-proteins and the ORCC, we immunodepleted CFTR from the immunopurified tracheal preparation prior to reconstitution of this material into the lipid bilayer. As previously shown, immunodepletion of CFTR did not affect the biophysical properties of the ORCC, although under these conditions the ORCC could not be activated by PKA + ATP (3). The addition of PTX to the presumed cytoplasmic face of the incorporated channel in the absence of CFTR resulted in a similar increase in P<sub>o</sub> of the ORCC as had been observed in the presence of CFTR (Fig. 4). Moreover, PTX restored sensitivity to PKA + ATP to the ORCC, as P<sub>o</sub> was increased from 0.63 ± 0.06 to 0.91 ± 0.08 (n = 12) under these conditions, even in the absence of CFTR (Fig. 4). Furthermore, PTX treatment also conferred a linearity to ORCC, as was the case in the presence of CFTR. I/V curves, derived from these experiments are shown in Fig. 5. It is clear from these plots that PKA-mediated phosphorylation of ORCC partially restored rectification properties after the addition of PTX. This effect of PTX was independent of the presence or absence of CFTR. GTPγS-treated ORCC, in contrast, was significantly more rectified at both negative and positive holding potentials (100 pS at +40 to +100 mV and 16 pS at −40 to −100 mV). Interestingly, the negative branch of the I/V curve of ORCC in the absence of CFTR was almost identical to the I/V curve of ORCC treated with GTPγS in the presence of CFTR.
FIG. 4. Effect of PTX and subsequent PKA phosphorylation on the immunopurified ORCC (in the absence of CFTR) incorporated into planar lipid bilayer. Holding potential, doses of PTX, PKA + ATP, DIDS, and DPC were as described for Fig. 3. Control refers to channel activity in symmetrical bathing solutions containing 100 mM KCl, 10 mM MOPS (pH 7.5). Additions were made sequentially as shown in the figure. The dashed line represents zero current level. Records were filtered at 100 Hz.

FIG. 5. Single channel current-voltage relationship of immunopurified and reconstituted ORCC in the presence and absence of CFTR before and after the addition of PTX. Conditions are defined in the symbol legends on the graph. Symbols indicate mean value, and error bars indicate ± S.D. for at least five separate experiments for each condition.

DISCUSSION

The results presented in this study show that a pertussis toxin-sensitive G-protein copurifies with bovine tracheal ORCC and CFTR channels and is involved in the direct regulation of the ORCC. We have previously demonstrated that solubilized apical membrane vesicles from bovine trachea, immunopurified with an antibody raised against an anion channel protein, contain both an ORCC and CFTR (3). The addition of PKA + ATP to the cis (or cytoplasmic) bathing solution of a bilayer containing this material activated a large (80 pS at +80 mV) conductance, outwardly rectified anion channel that was sensitive to DIDS. Inhibition of this 80-pS channel with 100 μM DIDS in the presence of PKA and ATP revealed a second, low conductance anion channel (16 pS) that exhibited a linear I/V relationship and that was sensitive to DPC. These data suggested that the immunopurified tracheal material contained both a functional ORCC and a CFTR. Moreover, these channels maintained a regulatory relationship even after this harsh purification procedure, e.g., the presence of CFTR was required for PKA activation of ORCC (3). In contrast, the recent model proposed to explain regulation of Cl⁻ transport via ORCC and CFTR in epithelial cells (31, 32) includes an amplification cascade initiated by an initial interaction of extracellular ATP with a G-protein-coupled P₂U receptor. Our studies with immunopurified proteins reconstituted into planar lipid bilayers demonstrated that the ORCC could be activated by PKA in the presence of G551D mutant CFTR, but only when ATP was added to both sides of the channel-containing bilayer, consistent with the external ATP stimulation part of this model (19).

Several other studies report copurification of channels or receptors with associated G-proteins (24, 27–29). In our preparation, a PTX-sensitive, ADP-ribosylated G-protein remained associated with the ORCC complex after CFTR precipitation. A direct regulatory relationship between the ORCC and a G-protein was confirmed by the addition of GTPγS or PTX to the ORCC either by itself or incorporated together with CFTR. The addition of pertussis toxin, which prevents the dissociation of the heterotrimeric G-protein complex, activated the ORCC. The addition of GTPγS decreased the activity of the ORCC in the planar lipid bilayer. Uncoupling of the ORCC from the G-protein also altered the rectification properties of the channel, suggesting that channel rectification is influenced at least in part by its interaction with a G-protein. Interestingly, the ORCC regained PKA sensitivity after the addition of PTX, even in the absence of CFTR. These results are consistent with the findings of Schwiebert et al. (18) that G-proteins are involved in the regulation of Cl⁻ transport in airway epithelia. It would appear that uncoupling of the effector (in our case ORCC) from its associated G-protein restores PKA sensitivity to the channel. It is known that phosphorylation of the β-receptor plays a role in desensitization of this receptor (30). Phosphorylation in this case prevents interaction between a G-protein and the phosphorylated β-adrenergic receptor and stops further mediation of signals from the stimulated β-receptor (30). A similar mechanism between the ORCC and a G-protein may also exist. Phosphorylation of the channel may uncouple a G inhibitory (Gι) protein from the channel and thus activate it. Dephosphorylation of the channel would be predicted to permit the Gi protein to interact with the channel again and to inhibit it. However, after activation with PTX (uncoupling of the channel from the G-protein; Ref. 31), additional stimulation with PKA and ATP was possible, suggesting that complete uncoupling of the channel from a G-protein may uncover additional phosphorylation sites on the channel protein. Moreover, these findings indicate that there are at least two major independent regulatory inputs that converge on the ORCC.

Recently, Schwiebert et al. suggested that ATP transported through CFTR acts as an autocrine stimulator of the ORCC (32). The proposed mechanism of regulation is via a P₂U receptor that, either through a direct coupling to the ORCC or through a G-protein-coupled signaling pathway, stimulates the ORCC. We have previously shown that the active form of CFTR is required for PKA-mediated activation of ORCC (19), while in the present study we have demonstrated the presence of a G-protein in our preparation. However, in contrast to the findings of Guggino and co-workers (32), namely that extracellular
ATP at nanomolar concentrations stimulates the ORCC in normal or CF cells, we found that under our experimental conditions ATP had no effect on channel activity if CFTR was not present in the membrane. Moreover, ATP had no effect on the ORCC if PKA + ATP was not present on the cytoplasmic side of incorporated channels. There are several possible explanations for our results. One possibility is that ATP (on the cis side of the bilayer) may bind to the ORCC, which subsequently interacts with CFTR and is thereby activated. Interaction of the ORCC with CFTR may allosterically diminish interaction of the ORCC with its G-protein, as both the G-protein (inhibition) and CFTR (activation) regulate the ORCC and have opposite effects on the channel. Rectification of the ORCC may be in part due to G-protein coupling, because treatment with PTX confers a linearity to the otherwise outwardly rectified behavior of the channel. CFTR also affects rectification, because when CFTR was present, the ORCC exhibited less rectification (i.e. G-protein coupling induces rectification, whereas CFTR decreases rectification).

A second possibility to consider is that ATP may bind to CFTR directly. Alternatively, a receptor for ATP (purinergic receptor) could be strongly associated with CFTR. If this hypothesis is correct, we may have copurified a purinergic receptor together with CFTR. However, it would seem unlikely that the appropriate signaling pathway would be maintained under our conditions. If, on the other hand, CFTR is both coupled to ATP and bound to a G-protein, ATP binding could uncouple the G-protein from its effector (in this case ORCC). This would allow the involvement of CFTR in the regulation of different processes through G-proteins. In either case, we have demonstrated that both a G-protein and CFTR are involved in the regulation of ORCC.

In summary, we have shown that a pertussis toxin-sensitive G-protein copurifies with ORCC and CFTR and that, after precipitation of CFTR, the G-protein remains in the material that contains the ORCC. The effects of PTX and GTPγS on the ORCC were independent of the presence or absence of CFTR. PTX and GTPγS did not have any effect on the CFTR channel activity under our experimental conditions (in absence of PKA and ATP from cytoplasmic side). These observations are consistent with the hypothesis that G-proteins directly regulate the ORCC, but not CFTR. Additionally, whereas we have previously demonstrated that precipitation of CFTR prevents the PKA + ATP-dependent activation of the ORCC (3), the data presented in this study have shown that even in the absence of CFTR, the ORCC can be activated by PKA + ATP as long as PTX is present on the cis side. This observation suggests a possible regulatory role of CFTR on the ORCC through a G-protein interaction.

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