Interaction between Cystic Fibrosis Transmembrane Conductance Regulator and Outwardly Rectified Chloride Channels*

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We have previously described a protocol for the simultaneous isolation and reconstitution of a protein kinase A (PKA)-sensitive outwardly rectified chloride channel (ORCC) and the cystic fibrosis transmembrane conductance regulator (CFTR) from bovine tracheal epithelium. Immunoprecipitation of CFTR from this preparation prevented PKA activation of the ORCC, suggesting that CFTR regulated the ORCC and that this regulatory relationship was preserved throughout the purification procedure. We now report the purification of CFTR from bovine tracheal epithelium and the purification of a CFTR conduction mutant (G551D CFTR) from retrovirally transduced mouse L cells using a combination of alkaline stripping, Triton-X extraction, and immunoaffinity chromatography. Immunopurified CFTR proteins were reconstituted in the absence and presence of ORCC. To test the hypothesis that only functional CFTR can support activation of ORCC by PKA and ATP, we used an inhibitory anti-CFTR505–511 peptide antibody or G551D CFTR. When anti-CFTR505–511 peptide antibodies were present prior to the addition of PKA and ATP, activation of both the ORCC and CFTR was prevented. If the antibody was added after activation of the ORCC and CFTR CI− channels by PKA and ATP, only the CFTR CI− channel was inhibited. When ORCC and G551D CFTR were co-incorporated into planar bilayers, only the ORCC was recorded and this channel could not be further activated by the addition of PKA and ATP. Thus, functional CFTR is required for activation of the ORCC by PKA and ATP. We also tested the hypothesis that PKA activation of ORCC was dependent on the extracellular presence of ATP. We added ATP on the presumed extracellular side of the lipid bilayer under conditions where it was not possible to activate the ORCC, i.e. in the presence of inhibitory anti-CFTR505–511 antibody or G551D CFTR. In both cases the ORCC regained PKA sensitivity. Moreover, the addition of hexokinase + glucose to the extracellular side prevented activation of the ORCCs by PKA and ATP in the presence of CFTR. These experiments confirm that both the presence of CFTR as well as the presence of ATP on the extracellular side is required for activation of the ORCC by PKA and ATP.

The cystic fibrosis transmembrane conductance regulator (CFTR) and outwardly rectified chloride channels (ORCC) both contribute to CAMP-activated chloride conductance in normal, but not in cystic fibrosis (CF), airway cells (1, 2). ORCCs are present in the apical membranes of CF airway epithelia, but they cannot be activated by protein kinase A (PKA) and ATP (3–5). CFTR channels are regulated by PKA and ATP and are inhibited by the channel blocker diphenylamine-2-carboxylate (DPC) but not by 4,4′-disothiocyanostilbene-2,2′-disulfonic acid (DIDS) (6, 7), whereas ORCCs are DIDS-sensitive. Although CFTR and ORCC channels are distinct proteins, they are linked through unknown regulatory pathways. Different mechanisms by which CFTR might regulate the ORCC can be envisioned. For example, CFTR could interact directly with the ORCC protein in the apical membrane, regulation could be a consequence of the channel function of CFTR, or both. It has been shown that CFTR can act as both an ATP and a Cl− channel (6). Recently Guggino’s group (7) demonstrated that ATP transported through CFTR acts as an autocrine stimulator of the ORCC. 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.

Increasing evidence suggests heterogeneity in the molecular pathogenesis of CF. Some mutations, such as the deletion of a phenylalanine at position 508 (ΔPhe-508) of CFTR, cause CFTR to be processed improperly within epithelial cells, and disease may result because the mutant protein fails to traffic to the apical cell membrane. Other mutations, such as the relatively common glycine → aspartic acid replacement at CFTR position 551 (G551D, 3% of all mutations), appear to be processed and targeted normally to the plasma membrane, but lack responsiveness to stimulation by cAMP (8). It is not known how different mutational forms of CFTR affect regulation of the ORCC by PKA.

Amino acid sequence analysis suggests that CFTR is composed of two motifs, each containing a membrane-spanning domain (MSD) and a nucleotide-binding domain (NBD) linked by a regulatory R domain (9). Recently, Welsh and colleagues (10) demonstrated that the amino-terminal portion of CFTR, which includes MSD1, NBD1, and the R domain, forms a regulated Cl− channel. It has been shown previously that an even smaller part of CFTR, namely NBD1 (amino acids 426–588), can form an anion channel when reconstituted in planar lipid bilayers (11). It is not known if these portions of the CFTR

* This work was supported in part by National Institutes of Health Grant DK 48764. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by Fellowship CFF981 provided by the Cystic Fibrosis Foundation.

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molecule can also transport ATP or if they are sufficient to replace intact CFTR in the regulation of the ORCC.

We recently reported the simultaneous isolation and functional reconstitution of an ORCC and CFTR from bovine tracheal epithelia (12). The regulatory relationship between these channel proteins was preserved throughout the purification procedure, as demonstrated by the activation of the ORCC by PKA and ATP. Immunoprecipitation of CFTR from this preparation prevented the activation of the ORCC by PKA and ATP, suggesting that the presence of CFTR is required for PKA-dependent activation of the ORCC. However, these experiments did not distinguish if functional CFTR was essential for conferring PKA sensitivity to the ORCC, if only the physical interaction of CFTR was required, or both.

We thus tested the hypothesis that the functional form of CFTR is required for PKA activation of the ORCC. In order to achieve this goal, we used an inhibitory anti-CFTR peptide antibody (13–15) to block CFTR transport function or replaced functional CFTR with a nonfunctional mutant form of CFTR, namely G551D CFTR (8). Our results indicate that only when CFTR is functional can the ORCC be activated by PKA and ATP. Our results also are consistent with the hypothesis of Schwiertz et al. (7) that the transport of ATP by CFTR to the extracellular side of the cells is necessary for PKA activation of the ORCC.

EXPERIMENTAL PROCEDURES

Materials

\( \gamma^3P \)ATP was obtained from DuPont NEN. A hydrazide-derivatized Acti-Disk was obtained from FMC Corp. (Pine Brook, N.J.). Peroxidase-free Triton X-100 and Bio-Beads SM2 were purchased 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.

Methods

Tracheal Apical Membrane Preparation—Bovine tracheal apical membrane vesicles were prepared by differential centrifugation using a procedure first described by Langridge-Smith et al. (16), modified as described previously (14). Aliquots of prepared 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 10.5 with 0.1 M NaOH. Alkaline-stripped vesicles were recovered by centrifugation at 35,000 × g for 35 min and then solubilized with Triton X-100 (0.8%) in the presence of KCl buffer (at pH 7.4). Quantification of solubilized protein was performed using the BCA method. Immunopurification of ORCC and CFTR—Channel Proteins with α38 Antibodies—Immunopurification of ORCC and CFTR was performed as described previously (12). A polyclonal rabbit antibody (α38) generated against the 38-kDa component of an anion channel protein was covalently linked to a hydrazide-activated disk. An equivalent amount of non-immune rabbit IgG was linked to another hydrazide-activated disk. Solubilized prewashed apical membrane vesicles were diluted in 40 ml of the above buffer and recirculated first over a non-immune disk and after that over the immune disk for 1 h (each disk) to allow unspecific and specific binding of the proteins. The disks were then extensively washed with NaCl/phosphate buffer before elution with 100 mM glycine, pH 3. Successive 2-ml fractions were collected and immediately neutralized with 40 μl of 1 M Tris. The first 15 fractions were pooled and concentrated to 500 μl and used for reconstitution in liposomes or for further biochemical characterization.

Immunopurification and in Vitro Phosphorylation of Normal and Mutant Form (G551D) of CFTR—Immunopurification of CFTR from bovine tracheal apical vesicles (wild type CFTR) or L cell lysate (G551D CFTR) was performed using polyclonal anti-CFTR\(_{\text{nond}}\) antibodies raised against a purified fusion protein corresponding to the first NBD1 (amino acids 426–588) of CFTR (18). L cell lysates were prepared as described previously (16). Essentially the same immunopurification procedure as described above with α38 antibodies was followed, except that solubilized proteins were precleared with non-immune IgG prior to recirculation over the disks and that the pH of the elution buffer was

2.7. Subsequent labeling and analysis of eluted proteins with \( \gamma^3P \)ATP was done as described previously (12). Complete solubilized material (30–40 μg of proteins) or immunopurified proteins (approximately 100–200 ng) were washed with reaction buffer (50 mM Tris, pH 7.5, 10 mM MgCl\(_2\)) and concentrated to 40 μl. PKA (300 ng) and 2 nmol of \( \gamma^3P \)ATP (3000 Ci/mmol) were added and the mixture incubated for 10 min at 30 °C. The reaction was stopped by adding SDS sample buffer containing 10 mM dithiothreitol. The samples were run on 6% acrylamide gels, and the dried gels were analyzed by autoradiography.

Immunoprecipitation of CFTR from α38 immunopurified bovine tracheal epithelial proteins—Immunoprecipitation of CFTR from α38-immunopurified bovine tracheal epithelial proteins was performed as described previously (19) using the polyclonal anti-CFTR\(_{\text{nond}}\) antibodies. Precipitation was followed by in radioimmunoprecipitation buffer that contained 1 mM Tris, pH 7.5, 550 mM NaCl, 1% Triton-X-100, 1% sodium deoxycholate, and 0.1% SDS to ensure dissociation of CFTR from other proteins.

Tissue Culture—Retrovirus transduced L cells containing G551D CFTR (Ref. 8; kind gift from Dr. Raymond Frizzell, University of Alabama at Birmingham), a murine fibroblast cell line, were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Production of Anti-CFTR\(_{\text{nond}}\) Antibody—New Zealand White rabbits were immunized with purified NBD1 (amino acids 426–588) fusion protein emulsified in Freund’s adjuvant (complete for initial immunization and incomplete thereafter) in a ratio of 1:1. Each animal received 400–500 μg of purified protein. The animals were bled from the marginal ear vein at 2-week intervals, and the sera were tested by an immunoblot assay as described previously (17), using the purified fusion protein as the antigen. Subsequently, the animals were bled and boosted as required.

Production of Anti-CFTR\(_{\text{nond}}\) Antibody—Anti-CFTR\(_{\text{nond}}\) antibody was produced as described in (13). New Zealand white rabbits were immunized with a synthetic peptide corresponding to amino acids 505–511 of CFTR. Site-directed antibody was affinity-purified from immune serum using synthetic 505–511 peptide coupled to CNBr-activated Sepharose. Whole cell patch clamp studies have shown that this antibody neutralizes CFTR function (13).

Polyacrylamide Gels—Protein separation on polyacrylamide gels was performed using the method of Laemmli (20). The apparent Mr of the unknown proteins was determined from appropriately constructed log Mr versus relative mobility (Rv) curves.

Plasmin Lipid Bilayer Experiments—Immunopurified Cl– channel proteins were reconstituted into liposomes as described previously (21). Briefly, concentrated protein samples (100–200 ng) were mixed with a phospholipid mixture (phosphatidylcholine/ammonium, phosphatidylserine, phosphatidylethanolamine, and oxidized phosphatidylcholine, 400/30/20, w/w/w, mixed at room temperature for 150 mg of Bio-Beads SM-2, first at room temperature for 45 min and then overnight at 4 °C). Proteoliposomes were separated from the beads using a 1-ml syringe fitted with a 27-gauge needle. Liposomes that contained co-reconstituted ORCC and G551D CFTR were produced by mixing an equal amount (100–200 ng) of immunopurified ORCC protein with immunopurified G551D CFTR protein and following the same procedure as for reconstitution of single immunopurified material.

Plasmin lipid bilayers, composed of a mixture of diphytanoyl-phosphatidylethanolamine, diphytanoyl-phosphatidylserine, and oxidized cholesterol in a 2:1:2 (w/w/w) ratio (final concentration = 25 mg/ml in n-octane), were painted with a fire-polished glass capillary over a 0.5-m hole drilled in a polystyrene chamber, as described previously (22). Bilayer formation was monitored by the increase in membrane capacitance to a final value of 300–400 picoFarads. Liposomes containing anion channel proteins were incorporated into bilayers bathed with symmetrical solutions of 100 mM KCl and 10 mM MOPS adjusted to pH 7.4. Channel incorporation was indicated by the appearance of quantal step increases in current with applied voltage. Current measurements were performed with a high gain amplifier circuit based on a design described previously (23). Single channel records were analyzed with pCLAMP, version 5.5. Steady-state single channel current–voltage (IV) curves were measured after channel incorporation by applying a known voltage and measuring individual channel current (i). Single channel open probability (Po) was calculated from Po = T/i, where i is the unitary current, T is the open time, and n is the total number of active channels.

Both n and i were estimated from all the points current amplitude histograms produced by pCLAMP software. The dashed line in the figures represents the zero current level (or close state). All
Functional CFTR Regulates ORCC

RESULTS

Immunopurification of Bovine CFTR from Tracheal Epithelia and Recombinant G551D CFTR from L Cell Lysate—Previously we reported an immunopurification procedure for the simultaneous isolation of both an ORCC and CFTR from bovine tracheal epithelia. Incorporation of isolated proteins demonstrated that both the channel proteins, as well as the regulatory relationship between them, were very well preserved (12). To further delineate the mechanism of interaction between the ORCC and CFTR, we immunopurified and reconstituted two different forms of CFTR. The immunopurification procedure was essentially the same as that previously described for the simultaneous isolation of the ORCC and CFTR, except that anti-CFTRNBD1 antibody was used. Mild conditions of immunopurification helped to preserve functional protein for reconstitution. The cell lysate of L cells expressing G551D served as a source for a nonfunctional, mutated form of CFTR. In vitro phosphorylation of immunopurified material revealed a dominant 170-kDa phosphoprotein isolated from either bovine tracheal epithelia (Fig. 1A, lane 2; n = 3) or L cell lysate (Fig. 1B, lane 2; n = 3). It was shown previously that the G551D form of CFTR is not different from wild type CFTR with respect to glycosylation (8). Our results indicate that anti-CFTRNBD1 antibodies are suitable for immunopurification of G551D CFTR from L cell lysates and that G551D CFTR can be in vitro phosphorylated.

Reconstitution of Bovine Tracheal CFTR and G551D CFTR—Equal amounts of immunopurified tracheal CFTR or G551D CFTR were each reconstituted into proteoliposomes and incorporated into planar lipid bilayers. Fig. 2A shows typical current traces of channel activity of bovine tracheal CFTR after incorporation and activation by PKA and ATP. No channel activity was observed whatsoever when incorporation was performed in the absence of PKA and ATP in the bathing solution. Incorporations were random; therefore, PKA + ATP was present in both compartments of the bilayer chamber prior to incorporation. When wild type CFTR-containing vesicles were used, a small (16 picosiemens) linear anion-selective channel was recorded. The identity of the incorporated channel was confirmed by its insensitivity to 100 µM DIDS, its sensitivity to DPC, and its block by anti-CFTRNBD1 antibodies (Fig. 2B). Moreover, this channel had a Cl⁻ > I⁻ perme selectivity (4:1). Anti-CFTRNBD1 antibodies added to the one side of the bilayer containing incorporated CFTR, to a final concentration of 25–50 ng/ml, inhibited bovine CFTR channel activity in the presence of PKA and ATP. However, due to the random orientation of the channel in the bilayer, in some of the experiments (11/28) these antibodies did not inhibit CFTR when added to one side of the system only. In these same experiments, addition of antibodies to the opposite side successfully inhibited channel activity. Addition of the anti-CFTRNBD1 antibody (the antibody used in the purification of CFTR) to either or both sides of the bilayer did not have any effect on the activity of incorporated bovine CFTR (Fig. 2). Incorporation of G551D CFTR and treatment with PKA and ATP did not result in the appearance of any channel activity (n = 15).

Effect of Inhibitory Anti-CFTRNBD1 Antibodies on Activation of the ORCC by PKA and ATP—We next examined whether inhibitory anti-CFTR antibodies could prevent the activation of ORCC by PKA + ATP. For these experiments we used immunopurified material obtained using p38 antibodies that contained CFTR co-purified with the ORCC. Current rectification of the ORCC allowed us to determine the sidedness of the incorporated channels. Moreover, the incorporation of proteins into negatively charged bilayers held at −80 mV permitted us to perform oriented reconstitutions. The cytoplasmic (PKA-sensitive) portion of both channels faced the cis compartment, while the extracellular portion faced the trans compartment in more than 90% of successful incorporations of ORCC + CFTR, as assessed by PKA activation. Another observation allowed us to determine whether CFTR was co-reconstituted into the bilayer membrane prior to PKA activation. Current voltage (IV) curves of ORCC in the presence of nonphosphorylated CFTR were slightly less rectified in the negative voltage quadrant compared with when CFTR was absent (Fig. 5). This distinction was important because in a few experiments when PKA + ATP failed to activate ORCC incorporated into the bilayer membrane (3 out of 72), using the criterion described above, we concluded that CFTR was not present in the bilayer. Furthermore, in these same three experiments, CFTR channel activity also was not observed subsequent to addition of PKA + ATP to either side of the bilayer.

We found that anti-CFTRNBD1 antibodies had no effect on base-line ORCC activity, confirming their specificity toward CFTR. If inhibitory anti-CFTRNBD1 antibodies were added to
The presumptive cytoplasmic bathing solutions after PKA and ATP activation of the incorporated channels, only CFTR Cl \(^{-}\) channel activity was inhibited, while ORCC remained activated with a \(P_F = 0.78 \pm 0.06\) (n = 21; Fig. 3A). Amplitude histograms of channel recordings made in the presence of PKA and ATP (Fig. 3B) showed that at least three CFTR Cl \(^{-}\) channels accompanied one ORCC channel (only one ORCC was active in the bilayer after inhibition of CFTR using anti-CFTR(505–511) antibodies). However, it would be premature to draw any conclusions about the exact molar ratio of ORCC and CFTR necessary for interaction from these bilayer experiments, because in other comparable experiments two to five CFTR channels per single ORCC have been observed. In contrast to these results obtained with anti-CFTR(505–511) antibodies added after PKA + ATP activation of incorporated channels, addition of inhibitory anti-CFTR(505–511) antibodies prior to addition of PKA and ATP prevented activation of both CFTR and ORCC by PKA + ATP (n = 11; Fig. 4, A and B).

Anti-CFTR(505–511) antibodies did not affect the rectification properties of ORCC, although by using these antibodies to block CFTR channel activity we were able to determine more precisely the I/V curves of phosphorylated ORCC. Notably, rectification of ORCC upon PKA-induced phosphorylation did not change. The slope conductance of ORCC at positive voltages was not altered under any conditions. These results support the hypothesis that the active form of CFTR is required for activation of ORCC by PKA and ATP.

Effect of Replacement of Functional CFTR with Nonfunctional G551D CFTR on Activation of ORCC by PKA and ATP—To test further the hypothesis that the functional form of CFTR is required for activation of the ORCC by PKA and ATP, we co-reconstituted immunopurified ORCC and nonfunctional G551D CFTR. Separation of ORCC from co-purified ORCC and CFTR was achieved by immunodepletion of CFTR from the preparation. The removal of CFTR was confirmed by the absence of a 170-kDa phosphoprotein and lack of activation by PKA + ATP of reconstituted ORCC incorporated into planar lipid bilayers, as described earlier (12). G551D CFTR was immunopurified as described under “Methods” and co-reconstituted with the preparation containing ORCC but devoid of CFTR. For replacement of normal CFTR with G551D CFTR we used autoradiography/densitometry to estimate the intensity of the in vitro phosphorylated CFTR (170 kDa) band and replaced normal CFTR with a similar amount of G551D CFTR, as G551D CFTR can also be in vitro phosphorylated. Under these conditions following incorporation into the bilayer, only the ORCC was recorded with no change in open probability upon addition of PKA and ATP to one (either cis or trans) side of the bilayer (80 mV in the presence of 100 \(\mu M\) DIDS and 100 \(\mu M\) ATP; Fig. 4, A and B). Detailed analysis of the electrical properties of ORCC revealed a change in its rectification properties in the presence of G551D CFTR in the negative potential quadrant, similar to what was described for ORCC in the presence of normal CFTR (see Fig. 5). In three separate experiments, liposomes containing only G551D CFTR were fused with a bilayer already containing ORCC but not CFTR. The presence of G551D CFTR, like incorporation of wild type CFTR, produced a change in the rectification properties of ORCC. In only 2 experiments from a total 26 performed, ORCC incorporated from liposomes presumably containing both ORCC and G551D CFTR displayed properties identical to ORCC incorporated from CFTR-immunodepleted preparations. These observations allowed us to conclude that the change of rectification was indicative of the presence of G551D CFTR in a bilayer containing ORCC. Nonetheless, the ORCC was not activated by PKA + ATP in these experiments (n = 9), thus supporting the hypothesis that the conductive form of CFTR was required for the activation of the ORCC by PKA and ATP. However, in three experiments in which PKA and ATP were added to both sides of a bilayer containing ORCC and G551D
CFTR, the activity of ORCC was increased 2-fold (from 0.38 ± 0.05 to 0.81 ± 0.07, data not shown).

Effect of Extracellular ATP on Activation of ORCC by PKA and ATP—It has been shown that CFTR is both a Cl⁻ channel (6). To test if the transport of ATP by CFTR is required for activation of the ORCC, we added an ATP-scapenging system, hexokinase (0.5 unit/ml) + glucose (5 mM), to the presumptive extracellular side of the bilayer containing incorporated ORCC and CFTR Cl⁻ channels. The presence of hexokinase + glucose on the presumptive extracellular side did not influence activation of the CFTR channel by PKA + ATP added to the opposite side of the bilayer, but it did prevent the PKA-mediated activation of ORCC (n = 12). These results are in contrast to the PKA-mediated activation of ORCC and CFTR seen in the absence of hexokinase and glucose (cf. Fig. 3).

The aforementioned results suggested that ATP secretion through CFTR may be required for the PKA-induced activation of the ORCC. Addition of ATP by itself from the presumptive extracellular side did not have any effect on the channel activity of the incorporated ORCC + CFTR. If transport of ATP through CFTR was the only requirement for PKA-mediated activation of the ORCC, it should be possible to activate ORCC by the addition of ATP to the extracellular solution and PKA + ATP to the cytoplasmic side of the system. However, when ORCC alone was incorporated into a bilayer, this channel could not be activated by addition of 100 μM ATP to the “outside” and PKA + ATP to the “inside” or by PKA + ATP to both bathing solutions (see Fig. 7 of Ref. 12). These results suggest that transport of ATP through CFTR is not the only role that CFTR plays in the regulation of the ORCC.

We therefore examined if the presence of ATP on the presumptive extracellular side of a bilayer containing ORCC and nonfunctional CFTR (i.e. either blocked with the inhibitory antibody or nonfunctional G551D CFTR) could restore PKA sensitivity to the ORCC. Under both conditions, namely, in the presence of inhibitory antibodies (Fig. 6) or G551D CFTR (Fig. 7), the ORCC regained PKA sensitivity when 100 μM ATP was added to the extracellular bathing medium. These findings also confirmed the hypothesis that transport of ATP is not the only role that CFTR plays in the regulation of ORCC. These results also indicate that G551D CFTR, like wild-type CFTR, incorporated into the membrane with a specific orientation in the presence of ORCC.

We also tested whether a portion of CFTR, namely the NBD1 domain, could interact with the ORCC in the presence of extracellular ATP and be sufficient for PKA-mediated activation of ORCC. However, in four separate experiments, we found that NBD1 could not substitute for full-sized CFTR in the regulation of ORCC by PKA and ATP added from either or both sides of the membrane.

DISCUSSION

Our results demonstrate that we have immunopurified and functionally reconstituted into planar lipid bilayers bovine CFTR and nonconductive G551D CFTR from transduced L cells. A similar immunopurification procedure for the isolation and reconstitution of recombinant, and functionally competent, CFTR from Chinese hamster ovary and Sf9 cells was reported recently (24). Our reconstituted proteins were used with purified and functionally reconstituted ORCC (as described previ-
Immunopurified and reconstituted bovine CFTR Cl\(^{-}\) channel had the same ion selectivity, inhibitor specificity, and inhibition by anti-CFTR\(_{505-511}\) antibodies as reported previously in patch clamp studies or for reconstituted recombinant CFTR (7, 13, 25). The main difference between the previously reported characteristics of the CFTR Cl\(^{-}\) channel and the bovine CFTR Cl\(^{-}\) channel was the single channel conductance. In our experiments the conductance of bovine CFTR was 16 picosiemens, somewhat larger than that previously reported (8–13 picosiemens). The conductance of the CFTR channel in our hands was the same regardless of the antibody that was used for purification or the presence or absence of the ORCC in the reconstituted material. The reason for this discrepancy is not apparent.

Our study demonstrated that only when the transport function of CFTR was intact could the activation of the ORCC by PKA and ATP occur. Our results also support Guggino’s (7) hypothesis that one function of CFTR in the activation of the ORCC is to transport ATP to the extracellular side of the cell membrane. This hypothesis is consistent with the findings of Reisin et al. (6) who have demonstrated directly that ATP can traverse the CFTR channel. We cannot at present exclude the co-purification of a purinergic receptor by our procedures. However, it is unlikely that a receptor-coupled mechanism would be active in the bilayer system because of the absence of necessary signaling molecules. However, the failure of ORCC to be activated by PKA + ATP in the presence of external ATP supports the idea that a purinergic receptor mediated pathway does not co-purify in our preparation. The second finding revealed by our study is that a nonconductive form of CFTR (G551D) was able to support activation of the ORCC by PKA when ATP was present on the extracellular side of the incorporated channels.
channels (10, 11). We tested whether NBD1 could substitute for CFTR in the regulation of the ORCC by PKA by ATP. Under the conditions used in these studies, NBD1 did not form a channel in the bilayer and therefore was not able to substitute for CFTR. Furthermore, a recombinant NBD1 purified from prokaryotic cells was unable to support activation by PKA of the ORCC in the presence of exogenously added extracellular ATP. Taken together these results suggest that there is a minimal size requirement of CFTR necessary to subserve its regulatory role in the PKA-related activation of the ORCC.

In summary, we have immunopurified and functionally reconstituted CFTR from bovine trachea and nonconductive G551D CFTR from transduced L cells. In combination with previously co-purified ORCC and CFTR from bovine tracheal epithelia, and inhibitory anti-CFTR505–511 antibodies, we were able to explore the regulatory relationship between the ORCC and CFTR. We have shown that functional CFTR is required for activation of the ORCC by PKA and ATP. CFTR is apparently required to transport ATP from the cytoplasm to the extracellular bathing solution, where it can then interact with some external domain of the ORCC. The observation that the ORCC cannot be activated by extracellular ATP and PKA + ATP added to the cytoplasmic side in the absence of the CFTR molecule also suggests that, in addition to the transport (ATP) function of CFTR, a direct interaction between CFTR and ORCC is essential to confer PKA sensitivity to the ORCC.

Acknowledgments—We thank Dr. Tamas Jilling for help in the production of CFTR sera, antibodies. We thank Deborah Keeton for excellent technical assistance and Charlae Starr for prompt secretarial help. We greatly appreciate the gift of purified catalytic subunit of protein kinase A from Dr. Gail Johnson (UAB).

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J. Biol. Chem. 1995, 270:29194-29200.
doi: 10.1074/jbc.270.49.29194

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