Purified Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Does Not Function as an ATP Channel*

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Canhui Li, Mohabir Ramjeesingh, and Christine E. Bear†
From the Division of Cell Biology, Research Institute of The Hospital for Sick Children and the Department of Physiology, University of Toronto, Toronto, Ontario, Canada MSG 1X8

The gene mutated in cystic fibrosis codes for the cystic fibrosis transmembrane conductance regulator (CFTR). Previously, we provided definitive evidence that CFTR functions as a phosphorylation-regulated chloride channel, reconstituted protein. Recent patch-clamp studies have lead to the suggestion that CFTR may also be capable of conducting ATP or inducing this function in neighboring channels. In the present study, we assessed the ATP channel activity of purified CFTR and found that the purified protein does not function as an ATP channel in planar bilayer studies of single channel activity nor in ATP flux measurements in proteoliposomes. Hence, CFTR does not possess intrinsic ATP channel activity and its putative role in cellular ATP transport may be indirect.

The function of CFTR2 (the cystic fibrosis transmembrane conductance regulator) as a phosphorylation and ATP-regulated chloride channel is thought to be critical for the elaboration of salt and water secretion across the epithelial cell lining of multiple organs; the airways, pancreatic ductules, gastrointestinal tract, and reproductive tract (1). The chloride channel activity of CFTR was confirmed using a number of experimental approaches. First, expression of recombinant CFTR in heterologous cell systems conferred the appearance of CAMP-regulated chloride channels (2, 3). Second, mutagenesis of amino acid residues thought to reside in membrane spanning domains caused alteration in single-channel conductance and/or anion selectivity of recombinant CFTR (4). Finally, reconstitution of purified CFTR in planar phospholipid bilayers causes the appearance of PKA and ATP-regulated chloride channels, exhibiting biophysical properties identical with those observed in patch clamp studies of epithelial cell membranes (5–7).

It is not yet clear whether defects in the chloride channel activity of CFTR can account for the diverse symptoms of cystic fibrosis. In vivo nasal potential difference measurements in CF patients revealed an altered sodium conductance as well as chloride conductance (8). It was recently shown in vitro studies that the activity of the epithelial sodium channel ENaC was inhibited by coexpression with CFTR (9). Further, it has been suggested that CFTR may interact with another type of chloride channel found in the apical membrane of epithelial cells, the outwardly rectifying chloride channel (ORCC). Activation of CFTR channel function by phosphorylation can stimulate activity of the ORCC (10, 11). Hence, CFTR may act to promote epithelial cell secretion not only by phosphorylating chloride channel activity but also through regulatory interactions with other ion channels located on the apical membrane.

Conceivably, there are several mechanisms through which CFTR may interact with other ion channels localized at the apical membrane of epithelial cells. Functional interaction between CFTR and other channels may be due to the generation of permissive electrochemical gradients, convergence of signal transduction systems, or through direct protein-protein interactions. Recently, it has been suggested that CFTR may affect the activity of ORCC through an “autocrine” mechanism. According to Guggino’s research group (12), CFTR may activate the ORCC by mediating the efflux of ATP which then acts to stimulate ORCC through interaction with neighboring purinergic (P2U) receptors. There are several observations which support this hypothesis. First, extracellular ATP has been shown to stimulate the ORCC channel by interacting with P2U receptors (13). Second, as previously mentioned, CFTR activation leads to the stimulation of the ORCC (10, 11). Third, several patch clamp studies have reported that CFTR, or an intimately associated ion channel, may be capable of conducting ATP (12, 14). These latter studies have generated considerable controversy as high, unphysiological, concentrations of ATP (100 mM) were used to assay currents. Furthermore, some research groups have recently reported conflicting patch clamp data which suggests that ATP cannot be conducted through CFTR (15).

In the present investigation, we sought to assess the intrinsic ATP channel activity of CFTR by examining the conductance properties of purified, reconstituted CFTR.

MATERIALS AND METHODS

CFTR Production and Purification—Two liters of SF9 cells were infected with recombinant baculovirus containing the complete coding sequence for CFTR as described previously (2). CFTR purification was performed according to our published procedure (5) with the following modifications. Infected cells were harvested after 48 h and the pellet was treated with a phosphate-buffered saline containing 2% Triton X-100, 10 units/ml DNase, 5 mM MgCl2, 2 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, and 10 μM E64. The mixture was stirred for 2 h at 15°C after which the pellets were centrifuged at 100,000 × g for 2 h. The resulting pellets were treated with 180 ml of 2% SDS, 2% mercaptoethanol in 10 mM sodium phosphate, pH 7.2, and the mixture was stirred overnight at 4°C. Insoluble material was centrifuged at 60,000 × g, and the supernatant was filtered through a 0.22-μm filter before being applied at 1 ml/min to a ceramic hydroxyapatite column. Washing, elution, and identification of the CFTR-containing peaks were performed as described previously (5). The CFTR-containing fractions from the ceramic hydroxyapatite column were

1 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; PKA, protein kinase A; ORCC, outwardly rectifying chloride channel; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylserine; LiDS, lithium dodecyl sulfate.

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† Medical Research Council Scientist of Canada. To whom correspondence should be addressed: Division of Cell Biology, Research Institute, Hospital for Sick Children, 555 University Ave., Toronto, Ontario MSG 1X8, Canada. Tel.: 416-813-5981; Fax: 416-813-5028; E-mail: bear@sickkids.on.ca.
concentrated and applied to a Superose 6 column as the final purification step. The purified protein was quantitated by amino acid analysis. N-terminal sequencing and amino acid analysis were performed by the Hospital for Sick Children-University of Toronto-Pharmacia Biotechnology Centre.

CFTR Reconstitution into Phospholipid Vesicles—The CFTR sample (5 μl) in 1 ml of solution containing 10 mM Tris buffer, pH 7.8, containing 0.25% LiDS and 100 mM NaCl was concentrated in a Centricon concentrator (molecular mass cutoff 10 kDa) to a volume of 100 μl. The protein sample was then diluted to 1000 μl with 8 ml Hepes buffer containing 0.5 mM EGTA, pH 7.2 (Buffer A), and reconstituted to a 100-μl volume. The final LiDS concentration was 0.025%. Liposomes, 10 mg/ml, were prepared in Buffer A as described previously using a lipid mixture of PE:PS:PC:ergosterol, 5:2:1:1 by weight. One hundred microliters of liposomes containing 1 mg of lipid was added to 100 μl of the CFTR sample yielding a final LiDS concentration of 0.0125%. The mixture was dialyzed (Spectrapor molecular mass cutoff 50 kDa) for 18 h against 2 liters of Buffer A containing 1.5% sodium cholate. Dialysis was then continued against 4 liters of Buffer A for 2 days with daily changes of buffer. For lipid bilayer studies, nystatin was introduced into the reconstituted proteoliposomes as described previously (5). For the "Garty" flux assay (16), purified CFTR was reconstituted into proteoliposomes containing a mixture of the phospholipids: PE:PS:PC:cholesterol (5:4:1:4).

Phosphorylation of CFTR—CFTR proteoliposomes and liposomes without CFTR, both in 50 mM Tris, 50 mM NaCl at pH 7.4 were mixed with 200 mM catalytic subunit of PKA, 20 μM ATP, and 5 mM MgCl2, briefly sonicated and incubated for 1 h at room temperature. These conditions mimic those used for in vitro phosphorylation of CFTR immunoprecipitated from CHO cells (18). To confirm that purified, reconstituted CFTR was phosphorylated under these conditions, [γ-32P]ATP was used in one experiment to phosphorylate CFTR. An autoradiograph of the phosphorylated protein run on 6% SDS-polyacrylamide gel (19) showed a single intense band corresponding to CFTR. In order to remove PKA after the phosphorylation reaction, CFTR proteoliposomes and control, protein-free liposomes were airfuged at 100,000 g for 30 min and then washed twice by sonication with buffer and pelleted in the airfugal. Proteoliposomes were resuspended in the appropriate buffer and assayed for ATP channel activity using bilayer and flux assays. For bilayer studies of channel function, CFTR was phosphorylated as described above. Subsequently, PKA was separated from the proteoliposomes by microspin chromatography using Sephadex G-50. To validate complete removal of PKA, we assessed the presence of [[32P]labeled catalytic subunit of PKA in the proteoliposome sample and determined that three cycles of sonication followed by separation on a microspin column were needed for complete removal of the labeled subunit of PKA.

Concentrate Tracer Uptake Assay for Study of CFTR Function—A concentrated tracer uptake assay developed by Garty et al. (16) and modified by Goldberg and Miller (17) was also used to characterize the conductance properties of reconstituted CFTR. Proteoliposomes were prepared with 150 mM KCl and centrifuged through Sephadex G-50 columns equilibrated with glutamate salts; K-glutamate (125 mM), Na-glutamate (25 mM), glutamic acid (10 mM), Tris-glutamate (20 mM) at pH 7.6, to replace external chloride. Uptake was initiated and quantitated by addition of 1.0 μCi/ml 35Cl– or [3H]ATP. Intraspecific 35Cl– or [3H]ATP was assayed at various time points using a mini anion-exchange column (Dowex 1) (16) to separate liposomes from external media.

Planar Bilayer Studies of Liposomes Containing Purified CFTR—Purified CFTR was reconstituted into proteoliposomes containing a phospholipid mixture PE:PS:PC:ergosterol (5:2:1:2) for bilayer studies. Nystatin (120 μg/ml) was introduced into the proteoliposomes for bilayer formation to promote proteoliposome/bilayer fusion and the detection of these fusion events (20). Fusion of nystatin-containing liposomes was indicated by the appearance of transient nystatin spikes in bilayer conductance. As in our previous experiments, a 10 mM solution of phospholipid (PE:PS at a ratio of 1:1, Avanti Polar Lipids) in n-decane was painted over a 200-μm aperture in a bilayer chamber to form bilayers. Bilayer formation was monitored microscopically by observing the increase in membrane capacitance. In all experiments, bilayer capacitance was greater than 200 pF. Fusion of liposomes was potentiated with the establishment of an osmotic gradient across the lipid bilayer. The cis compartment of the bilayer chamber, defined as that compartment to which liposomes were added, contained 300 mM KCl and the trans compartment, connected to ground, contained 50 mM KCl. Single-channel currents were monitored at a holding potential of –40 mV and detected with a bilayer amplifier (custom made by M. Shen, Physics Laboratory, University of Alabama). Data were recorded and analyzed using pCLAMP 6.02 software (Axon Instruments, Inc.). Prior to analysis of open probability, single channel data were digitally filtered at 100 Hz.

RESULTS AND DISCUSSION

In order to directly assess the capacity of CFTR to conduct ATP, we first compared the single channel activity of purified CFTR reconstituted in planar lipid bilayers in the presence of symmetrical chloride (140 mM KCl) solutions with activity detected in the presence of symmetrical K2-ATP (140 mM) solutions. In all of these experiments, CFTR was phosphorylated by the addition of purified catalytic subunit of PKA plus MgATP and separated from these reagents by gel filtration (see "Materials and Methods") prior to reconstitution in lipid bilayers. Following the fusion of proteoliposomes with lipid bilayers, 1 mM MgATP was added to both the cis and trans compartments of the lipid bilayer chamber in order to activate channel function.

In the upper panel of Fig. 1A, we show activity of two CFTR channels in the presence of symmetrical concentrations of chlo-
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**Fig. 2.** Electrogenic 36Cl^- uptake mediated by CFTR-containing liposomes. A, the Garty 36Cl^- flux assay. This diagram shows the principle underlying the “Garty” flux assay of chloride and ATP conductance through purified CFTR reconstituted in liposomes. Proteoliposomes are loaded with KCl (150 mM), and external Cl^- was replaced with glutamate by rapid buffer exchange on a Sephadex G-50 “spin-column” to establish a large outward Cl^- gradient. Chloride-permeable proteoliposomes will become polarized (positive inside) and 36Cl^- added to the external medium will be taken up by the liposomes. B, time course of electrogenic 36Cl^- uptake by liposomes containing phosphorylated CFTR. The 36Cl^- uptake at 30 min after the establishment of the outward Cl^- gradient for Cl^- channel steps, confirming that CFTR is anion-selective and cannot pass gluconate. The subsequent substitution of K-glucuronate (100 mM) solutions eliminated the appearance of channel steps, confirming that CFTR is nonrectifying and its unitary conductance is 10 pS, consistent with previous reports of the nonrectifying I-V relationship of CFTR chloride channel activity. On the other hand, in the presence of asymmetrical anion solutions (KCl versus (K2-ATP)), single channel currents were only evident when the applied electrical potential (+40 mV) drove chloride current from the trans to the cis compartment. Single channel currents were not evident upon application of an electrical potential (-40 mV) which should drive ATP^- current from the cis to the trans compartments. These results support the data presented in Fig. 1 and suggest that CFTR cannot conduct ATP^-.

Further, it is clear from the single channel chloride current record obtained at +40 mV that the presence of K2-ATP in the trans compartment of a bilayer lipid vesicle results in a new channel open probability of 0.26 relative to control open probability of 0.68 measured in the presence of symmetrical KCl solutions. As in the preceding bilayer study shown in Fig. 1A, high ATP concentrations (100 mM) appear to inhibit CFTR channel function. On the other hand, there was no change in the amplitude of the chloride current step which occurs when CFTR opens, suggesting that ATP^- did not modify the conductive pathway for chloride ion.

It is conceivable that ATP^- may be conducted through CFTR but at a rate which is too slow for detection in planar lipid bilayer studies of single channel activity, we utilized the “Garty” method for assay of electrogenic anion flux anion in liposomes containing purified chloride channel protein (15, 16). Briefly, this assay, as modified by Miller et al. (18), involves the creation of an outward concentration gradient for chloride movement across proteoliposomes containing CFTR. The outward movement of chloride creates a positive potential inside the vesicle, thereby generating an inward driving force for chloride conductance, quantifiable by 36Cl^- addition (Fig. 2A). We show in Fig. 2B, that as predicted on the basis of existing single-channel data, liposomes containing PKA-phosphorylated CFTR exhibit significantly greater concentrative uptake of 36Cl^- than liposomes containing nonphosphorylated CFTR (p < 0.001).

We then compared the concentrative uptake of 36Cl^- and [3H]ATP by proteoliposomes possessing an inwardly-directed gradient for anion conductance. Concentrative uptake was determined at 30 min after the establishment of the outward chloride gradient, an interval close to the time determined for half-maximal uptake of 36Cl^- by phosphorylated CFTR. 36Cl^- uptake by CFTR-free liposomes and by liposomes containing unphosphorylated CFTR showed no significant difference (p = 0.327), confirming previous studies showing that unphosphorylated CFTR is not functional as a chloride channel (2–7). 36Cl^- uptake by PKA-phosphorylated CFTR was approximately 5-fold greater than that measured in liposomes with unphosphorylated CFTR. On the other hand, [3H]ATP^- taken up by the same liposomes showed no dependence on CFTR protein. At 30 min, [3H]ATP^- uptake by CFTR-free liposomes and liposomes containing phosphorylated CFTR was not significantly different (Fig. 3). These results suggest that CFTR does not mediate the conductance of [3H]ATP^-.

In summary, our results show that purified, reconstituted CFTR cannot conduct ATP^-.

As described, some previous patch clamp studies reported that CFTR expression is associated with ATP^- channel activity. This observation formed the basis for the “autocrine” model for CFTR regulation of the outwardly rectifying chloride channel (13). According to this model, ORCC channels are stimulated via G protein coupled to purinergic receptors (P2U) which are activated by ATP conductance through CFTR. The present studies show that purified CFTR cannot conduct ATP^- and we suggest that certain features of the autocrine model should be modified. For example, while we have shown that CFTR is not an ATP channel, we cannot exclude the possibility that CFTR may mediate ATP flux through a nonelectrogenic transport mechanism. This putative mechanism would explain existing data generated in cell lines wherein nonepithelial cells transfected with wild-type CFTR exhibited enhanced radiolabeled ATP efflux relative to cells which didn’t express CFTR or variant forms of CFTR known to cause disease in humans (12).
However, patch clamp data showing ATP currents associated with CFTR expression could not be reconciled with such a mechanism. Alternatively, it is conceivable that ATP conductance may be mediated through a channel which is functionally coupled to activated CFTR. The presence or absence of such functional interactions may account for some of the variability which exists between different patch clamp studies of the putative ATP channel activity of CFTR (12, 14, 15).

Finally, it is clear that the mechanism through which CFTR participates in cellular ATP transport and the role of this transport process in epithelial cell fluid secretion cannot be entirely defined in studies of purified, reconstituted CFTR protein alone. However, we have shown the utility of this reconstitution system in identifying those functions which are intrinsic to CFTR and those activities which are not intrinsic and may require interactions with neighboring proteins.

REFERENCES

1. Welsh, M., Tsui, L.-C., Baxt, T. F., and Beaudet, A. L. (1995) in The Metabolic Basis of Inherited Disease (Scriver C. R., Beaudet, A. L., Sly, W. S., and Vallee, D., eds) 7th Ed., pp. 3799–3876, McGraw-Hill Inc., New York
2. Kartner, N., Hanrahan, J., Jensen, T., Naismith, L., Sun, S., Ackerley, C., Reyes, E., Tsui, L.-C., Rommens, J., Bear, C., and Riordan, J. (1991) Cell 64, 681–691
3. Anderson, M., Gregory, R., Thompson, S., Souza, D., Paul, S., Mulligan, R., Smith, A., and Welsh, M. (1993) Science 253, 202–205
4. Tabcharani, J., Rommens, J., Hou, Y., Chang, X.-B., Tsui, L.-C., Riordan, J., and Hanrahan, J. (1993) Nature 366, 79–82
5. Bear, C., Li, C., Kartner, N., Bridges, R., Ramjeesingh, M., and Riordan, J. (1992) Cell 68, 809–818
6. Bear, C., and Reyes, E. (1992) Am. J. Physiol. 262, C251–C256
7. Tabcharani, J., Lowe, W., Elie, D., and Hanrahan, J. (1990) FEBS Lett. 270, 157–164
8. Knowles, M. R., Clarke, L. L., and Boucher, R. C. (1991) N. Engl. J. Med. 325, 533–538
9. Stutts, M. J., Canessa, C. M., Olsen, P. J., Hamrick, M., Cohn, J. A., Rossi, B. C., and Boucher, R. C. (1995) Science 269, 847–850
10. Egan, M. E., Flotte, T., Aflone, S., Sadow, R., Zettin, P. L., Carter, B. J., and Guggino, W. B. (1992) Nature 358, 581–584
11. Gabriel, S. E., Clarke, L. L., Boucher, R. C., and Stutts, M. J. (1993) Nature 363, 263–266
12. Schweibert, E. M., Egan, M. E., Hwang, T.-H., Pulver, S. B., Allen, S. S., Cutting, G. R., and Guggino, W. B. (1995) Cell 81, 1063–1073
13. Stutts, M. J., Fitz, J. G., Paradiso, A. M., and Boucher, R. C. (1994) Am. J. Physiol. 267, C1442–C1451
14. Reisin, I. L., Prat, A. G., Abraham, E. H., Amara, J. F., Gregory, R. J., Ausiello, D. A., and Cantiello, H. F. (1994) J. Biol. Chem. 269, 20584–20591
15. Grygorczyk, R., Tabcharani, J. A., and Hanrahan, J. W. (1996) Biophys. J. 70, A71
16. Garty, H., Rudy, B., and Karlish, S. J. D. (1983) J. Biol. Chem. 258, 13094–13099
17. Goldberg, A. F. X., and Miller, C. (1991) J. Membr. Biol. 124, 199–206
18. Seibert, F., Tabcharani, J., Chang, X., Dulhanty, A., Mathews, C., Hanrahan, J., and Riordan, J. (1995) J. Biol. Chem. 270, 2158–2162
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Woodbury, D., and Miller, C. (1990) Biophys. J. 58, 833–839