Electrodifusional ATP movement through the cystic fibrosis transmembrane conductance regulator

HORACIO F. CANTIELLO,1,2 GEORGE R. JACKSON, J R.,2 CLAUDIO F. GROSMAN,3 ADRIANA G. PRAT,1,2 STEVEN C. BORKAN,4 YIHAN WANG,1 IGNACIO L. REISIN,3 CATHERINE R. O'RIORDAN,5 AND DENNIS A. AUSIELLO1,2

1Renal Unit, Massachusetts General Hospital East, Charlestown 02129; 2Department of Medicine, Harvard Medical School, Boston 02115; 4Renal Section, Boston Medical Center, Boston 02118; 5Genzyme Corporation, Framingham, Massachusetts 01701; and 3Departamento de Química General e Inorgánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, 1113 Buenos Aires, Argentina

Cantiello, Horacio F., George R. Jackson, Jr., Claudio F. Grosman, Adriana G. Prat, Steven C. Borkan, YihAN Wang, Ignacio L. Reisin, Catherine R. O'Riordan, and Dennis A. Ausiello. Electrodifusional ATP movement through the cystic fibrosis transmembrane conductance regulator. Am. J. Physiol. 274 (Cell Physiol. 43): C799–C809, 1998.—Expression of the cystic fibrosis transmembrane conductance regulator (CFTR), and of at least one other member of the ATP-binding cassette family of transport proteins, P-glycoprotein, is associated with the electrodifusional movement of the nucleotide ATP. Evidence directly implicating CFTR expression with ATP channel activity, however, is still missing. Here it is reported that reconstitution into a lipid bilayer of highly purified CFTR of human epithelial origin enables the permeation of both Cl− and ATP. Similar to previously reported data for in vivo ATP currents of CFTR-expressing cells, the reconstituted channels displayed competition between Cl− and ATP and had multiple conductance states in the presence of Cl− and ATP. Purified CFTR-mediated ATP currents were activated by protein kinase A and ATP (1 mM) from the “intracellular” side of the molecule and were inhibited by diphenylamine-2-carboxylate, glibenclamide, and anti-CFTR antibodies. The absence of CFTR-mediated electrodifusional ATP movement may thus be a relevant component of the pleiotropic cystic fibrosis phenotype.

MATERIALS AND METHODS

Purification of CFTR. Recombinant human epithelial CFTR was obtained from baculovirus-infected SF9 (insect) cell membranes according to O'Riordan et al. (14). Briefly, CFTR was purified with a small (2.6 × 6 cm, 30 ml) ceramic hydroxypatite column and an equilibration buffer containing 10 mM sodium phosphate buffer, pH 6.4, 0.15% sodium dodecyl sulfate (SDS), and 5 mM dithiothreitol (DTT) and was eluted with a linear gradient of 100–600 mM sodium phosphate, 0.15% SDS, and 5 mM DTT as originally described (3). A Superdex column and chromatography in 0.1% SDS, rather than 0.25% lithium dodecyl sulfate, were used to facilitate purification of larger quantities (mg) of insect CFTR (14). In cases in which purified CFTR was obtained from CFTR-expressing Chinese hamster ovary (CHO) cells, cells grown on microcarriers were harvested, washed with sodium phosphate-buffered saline (PBS), and lysed in buffer containing protease inhibitors (for more details, see Ref. 14). The supernatant was processed to obtain a membrane suspension, which was pelleted at 100,000 × g for 60 min. The supernatant containing the solubilized CFTR was further purified by immunonoaffinity resins prepared with monoclonal antibody (MAB) 13-1 and MAB 24-1 (Genzyme, Framingham, MA), activated with sodium periodate, and coupled to Hydrazide Avidged (Unisyn Technologies) in 50 mM sodium acetate, pH 5.0. Immunonoaffinity chromatography was performed by incubation of CFTR-containing membranes (25 ml) with 15 ml of resin for at least 3 h at 4°C. After collection of the flow-through, the resin was rinsed with 100 ml of wash buffer containing 150 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane (Tris)·HCl, pH 8.0, 1 mM EDTA, and 1% sodium cholate to remove nonspecifically bound proteins. CFTR was further eluted from the resin using elution buffer (150 mM

THE CYSTIC FIBROSIS transmembrane conductance regulator (CFTR) supports ATP-dependent Cl− channel activity (3, 14). However, controversy still exists as to whether this functional feature of CFTR is sufficient to explain the complete pathophysiology of this disease (7). The cellular epithelial cystic fibrosis (CF) phenotype, for example, is associated both with a dysfuncional phospholipase A2 activity and a deranged arachidonic acid metabolism (10, 24) and with an abnormally increased apical epithelial Na+ permeability (6), which is regulated by CFTR (26). Expression of CFTR is also associated with the recovery of outwardly rectifying Cl− channel activity of CF cells (12) by an autocoid mechanism entailing the release of cellular ATP, which, in turn, helps modulate other cell responses (23). These disparate observations are unlikely to stem from an altered Cl− conductance. Alternate paradigms to explain the various features of the CF phenotype are currently being sought.

CFTR has been implicated in the release of cellular ATP (18, 23) and electrodifusional ATP movement (17, 22, 23); however, other studies have failed to detect electrodifusional ATP movement in preparations containing a functional CFTR (13, 21). In this report, we have revisited the question of whether CFTR is capable of permeating ATP. Highly purified and functional human epithelial recombinant CFTR (14) was reconstituted into a lipid bilayer in which addition of protein kinase A (PKA) and ATP elicited diphenylamine-2-carboxylate (DPC)-inhibitable Cl− and ATP-permeable ion channel activity, in agreement with our previous findings in intact cells (9, 22, 23). The present data indicate that ATP transfer by CFTR may not require an “adjacent” transmembrane structure to allow permeation of the nucleotide.
and reconstituted using a modification of the procedure in which affinity-purified CFTR was concentrated as described above. In cases in which ion exchange chromatography was performed on a Superdex 200 HR 10/30 column preequilibrated with 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 10% glycerol, and 0.5% sodium cholate, pH 7.5. Immunoaffinity-purified CFTR (200 µl) was applied to the column, fractions were collected, and CFTR was detected by SDS-polyacrylamide gel electrophoresis followed by silver staining. These fractions were pooled and concentrated, and CFTR was quantitated by an enzyme-linked immunosorbent assay (for details, see Ref. 14). In cases in which ion exchange chromatography was used, a 50-ml column of DEAE-Sepharose was equilibrated with 10 mM K₂HPO₄, 10% glycerol, and 0.05% α-lysophosphatidylcholine, pH 7.5. Stripped membranes (100 mg) were solubilized in the above buffer, and the resulting 100,000-g supernatant was applied to the column. CFTR bound to DEAE-Sepharose under these conditions. After extensive washing of the resin with equilibration buffer, a linear gradient of 10–150 mM K₂HPO₄ was applied. CFTR eluted from the column at ~60–85 mM K₂HPO₄ (14).

Assessment of CFTR purity. Purified CFTR obtained from insect SF9 cells was subjected to 4–20% SDS gradient gel electrophoresis to identify potential protein contaminants ranging in size from <1 to 300 kDa. Electrophoresis was stopped before the bromophenol blue dye front reached the bottom of the gel. The gel was then overlayed with silver to enhance sensitivity. Molecular size was estimated using markers obtained from GIBCO BRL (Grand Island, NY). Detection of CFTR by immunoblot analysis. Mouse mammary carcinoma (C127) cells transfected with human epithelial CFTR (WT1 cells) were harvested by washing with ice-cold Ca²⁺-free PBS (5 ml), scraped with a rubber policeman, centrifuged, and resuspended in ice-cold lysis buffer [containing 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM EDTA, 0.25 mM sodium vanadate, 10 µg/ml phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin] and then sonicated. Purified CFTR from SF9 cells was prepared as indicated above. Protein determination was performed using the Bio-Rad assay (Bio-Rad, Richmond, CA). Briefly, each sample was mixed with dilution buffer (final concentrations 62.5 mM Tris-HCl, pH 6.5, 2% SDS, 10% glycerol, and 50 mM DTT) and heated for 5 min at 60°C. Proteins were separated on a 7.5% SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride membranes premixed with methanol and soaked in transfer buffer for 15 min. Membranes were blocked with 5% dried milk and 0.5% nonimmune goat serum in TBST (50 mM Tris-HCl, pH 7.6, 141 mM NaCl, and 0.2% Tween 20) for 1 h. Blots were probed with one of two CFTR MAb (0.5–1 µg/ml; MAb 13-1 or MAb 24-1, Genzyme) in TBST containing 1% bovine serum albumin for 36–48 h at 4°C. After three washings with TBST, membranes were incubated with peroxidase-labeled goat anti-mouse antibody (Sigma) for 1 h at 25°C. Immunoreactive bands were detected by enzyme-linked chemiluminescence (Kirkegaard and Perry, Gaithersburg, MD).

Reconstitution of CFTR into phospholipid vesicles. Immunooaffinity-purified CFTR was concentrated as described above and reconstituted using a modification of the procedure described by Bear et al. (3). An aliquot containing a known amount of CFTR (15–20 µg/ml) was added to 100 µl of a solution containing 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.4) and 0.5 mM EGTA and also containing 1 mg of a sonicated phospholipid mixture of phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and ergosterol (5:2:1:2 molar ratio) and 1% sodium cholate. After a 40-min incubation on ice, the mixture was dialyzed at 4°C for 24 h against 15 mM HEPES, 0.5 mM EGTA, pH 7.4, and 1.5% sodium cholate. Dialysis continued with daily changes of buffer for an additional 3 days against the same buffer without sodium cholate. The sample was further dialyzed against 15 mM HEPES, 0.5 mM EGTA, and 150 mM sodium isethionate, pH 7.4, for 24 h. The resulting proteoliposomes were quickly frozen at –80°C, thawed, and sonicated for 5 s in a bath sonicator (Lab Supplies, Hicksville, NY). In some cases, reconstitution of CFTR-containing proteoliposomes was conducted without freezing and thawing to avoid the potential denaturing of the protein.

Planar lipid bilayer studies. Purified CFTR from either transfected SF9 or CHO cells was reconstituted into a lipid bilayer reconstitution system that has been previously used to assess CFTR-mediated Cl⁻ channel activity (3, 14). The reconstituted protein was either fused into the lipid bilayer by reconstitution of proteoliposomes with techniques previously described (3, 14) or applied directly to the lipid bilayer after elution through a Sephadex G-50 (fine) column to remove denaturing SDS. Reconstitution of CFTR under these conditions was conducted as follows. The glass rod used for painting the lipid bilayer was “dipped” consecutively in the CFTR-containing eluent and then into the lipid mixture used for forming the bilayer. This CFTR-containing mixture was used to break and repaint the lipid bilayer. To test the lack of detergent contamination as a potential source of “spurious” ion channel activity, equivalent samples of SDS were also eluted through a Sephadex G-50 column and/or incorporated into the bilayer as indicated above. None of the eluate SDS fractions tested showed a “nonspecific” leak conductance, either in the absence or presence of PKA. Thus reconstituted CFTR channel activity was only correlated with the presence of the protein and not with either contaminant SDS or protein degradation products that eluted before the protein. Experiments were also conducted with membrane preparations of CFTR-expressing WT1 cells (22) (data not shown). Either proteoliposomes containing purified CFTR or CFTR mixed with lipid components was fused to planar lipid bilayers by painting onto a 0.1-mm hole in a 13-mm polyethylene cuvette (Warner Instrument, Hamden, CT), as described by Alvarez (2). The phospholipid composition of the lipid bilayers was 1-palmitoyl-2-oleyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine, and oleyl-sn-glycero-3-phosphatidylserine (7:2:1, vol/vol/vol; Avanti Polar Lipids, Alabaster, AL) in n-decane (Aldrich Chemical, Milwaukuee, WI) to a final concentration of 14, 6, and 0.5 mM, respectively. CFTR eluted through Sephadex G-50 (fine) was collected at a final concentration of 1.0 µg/ml in saline. The initial ds and trans solutions usually contained, respectively, 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-20 µM CaCl₂ (pH 7.0) and 100 mM MgATP (pH 7.0). However, the solutions varied for the different experiments as indicated below.

Single-channel recordings of purified CFTR. Input signals were acquired with a PC-501A patch/whole cell clamp amplifier via a 10-GΩ head stage for lipid bilayers (Warner Instrument). The output currents were low-pass filtered at 500 Hz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA), digitized at 37 kHz and 14 bits with a VR-10B digital data recorder (Instrutech, Great Neck, NY), and...
stored on a video cassette recorder (JVC, Fairfield, NJ) until further analysis with pCLAMP 6.0.3 (Axon Instruments, Foster City, CA). Single-channel tracings were Gaussian-filtered at 100–200 Hz for display purposes, and analyzed as per patch-clamp protocols. Unless otherwise stated, values of n given in the text are the numbers of individual current measurements and do not necessarily reflect the numbers of experiments analyzed.

Calculation of the perm-selectivity ratio under asymmetrical ATP/Cl− conditions. To calculate the ATP-to-Cl− perm-selectivity ratio (P_{ATP}/P_{Cl−}) under asymmetrical conditions (ATP/Cl−), single-channel conductances γ [γ = current (I) divided by holding potential (V_h)] were best fitted to the Goldman-Hodgkin-Katz (GHK) equation, such that

\[ I(V_h) = \left( \frac{z_i F P_i V_h / RT}{1 - \exp(-\alpha)} \right) + \left( \frac{z_j F P_j V_h / RT}{1 - \exp(-\beta)} \right) \]

where i (species in trans compartment) and j (species in cis compartment) represent Cl− or ATP, depending on their locations on either side of the membrane. V_h is in mV, z and F are the charges for species i and j, respectively, and C_i and C_j are the concentrations of i and j, respectively. P_i and P_j represent the permeability coefficients for the species i or j, respectively, and α = z_i F V_h / RT and β = z_j F V_h / RT, where F, R, and T are Faraday’s constant, the gas constant, and absolute temperature, respectively. All cases in which MgATP was used were fitted with z = -2, and those in which Tris-ATP was used instead were fitted with z = -4.

Drugs and chemicals. ATP salts (MgATP and Tris-ATP; Sigma) were added from stock solutions (100 mM) in distilled water. The catalytic subunit of the adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase (PKA; Sigma) was used at final concentrations ranging from 75 to 252 nM. The Cl− channel blocker DPC (Fluka Chemical, Ronkonkoma, NY) was kept in a 100-fold stock solution (20 mM) in 50% water-ethanol. 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (Sigma) and glibenclamide (RBI, Natick, MA) were kept in 10 mM stock solutions in 100% dimethyl sulfoxide. The Mab raised against amino acids 729–736 of the regulatory domain (R-domain) of CFTR (MAb 13-1, Genzyme) was directly diluted 1:100 in the intracellular or bathing solution from a stock solution (292 µg/ml). An inactive antibody was obtained by preheating the CFTR antibody for 30 min at 100°C.

Table 1. Observation of channel activity in various CFTR preparations

<table>
<thead>
<tr>
<th>cis/trans Concentrations, mM</th>
<th>No. of Expts. with Channels</th>
<th>%Success</th>
<th>Comment</th>
</tr>
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<tbody>
<tr>
<td>50 KCl/100 MgATP</td>
<td>8</td>
<td>94</td>
<td>Sephadex purified and proteoliposomes</td>
</tr>
<tr>
<td>75 MgCl2/100 Tris-ATP</td>
<td>7</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>5–10 MgATP/300 NMGCl</td>
<td>29 of 52</td>
<td>56</td>
<td>Proteoliposomes</td>
</tr>
<tr>
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<td>7 of 16*</td>
<td>44</td>
<td>Sephadex purified</td>
</tr>
<tr>
<td>10 MgATP/100 MgATP</td>
<td>16 of 25</td>
<td>64</td>
<td>Sephadex purified</td>
</tr>
<tr>
<td>10 MgATP/100 Tris-ATP</td>
<td>9 of 9</td>
<td>100</td>
<td>Sephadex purified</td>
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<tr>
<td>10 MgATP/100 MgATP</td>
<td>4 of 4*</td>
<td>100</td>
<td>101 membranes</td>
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Statistics for successful experiments in which Cl− and/or ATP channel activity was observed for various cystic fibrosis transmembrane conductance regulator (CFTR) preparations. *Experiments in which sole presence of ATP and protein kinase A was sufficient to activate CFTR. NMGCl, N-methyl-o-glucamine chloride.

RESULTS

Cl− and ATP currents of highly purified human epithelial CFTR. To assess the role of CFTR in electrodiffusional ATP transfer, human epithelial CFTR was expressed and purified from either Sf9 insect or mammalian CHO cells (14) and functionally reconstituted into a lipid bilayer system to measure ion channel activity as reported previously (3, 14).
data of the various experimental conditions and preparations used are shown in Table 1. Purified material from either CHO (data not shown) or SF9 cells (Fig. 1) (14) was reconstituted in a chamber containing either 50 mM KCl and 100 mM MgATP (data not shown) or 75 mM MgCl₂ and 100 mM Tris-ATP (Fig. 2A) in the cis and trans compartments, respectively. Both compartments contained 10 mM MOPS, pH 7.4, and the cis compartment also contained 200 nM PKA. Eight of nine experiments in KCl/MgATP and seven of nine experiments in MgCl₂/Tris-ATP displayed anion-selective channel activity after PKA addition from the cis side of the chamber. Under the latter conditions, currents were observed in both directions (Fig. 2A), with at least two distinct (discernible) single-channel conductances of 8.57 ± 0.16 pS (n = 10) and 34.3 ± 0.32 pS (n = 7), respectively (Fig. 2B). The reversal potentials (Eᵥ) were −2.17 ± 1.83 mV (n = 10) and −0.35 ± 0.29 mV (n = 7) and were thus consistent with a Pᵥ/PCl of 0.1 and 0.2, for the smaller and larger conductances, respectively. These values are in close agreement with the Pᵥ/PCl values previously obtained from whole cell and single-channel currents of CFTR-expressing cells and calculated from the respective conductances with symmetrical concentrations of the two ions, in the range of 0.2–0.4 (22). A further indication that the CFTR molecule itself may be implicated in ATP transport is suggested by the observation that addition of the active, but not the heat-inactivated, anti-CFTR R-domain antibody 13-1 (Genzyme) inhibited simultaneously and almost completely both Cl⁻ and ATP currents of highly purified CFTR (Fig. 2C) in five of five experiments tested. These data further suggest that CFTR incorporation into the lipid reconstitution chamber was indeed functional and oriented as expected, with the PKA activation sites on the cis side of the membrane.

Single-channel currents seemed to correspond to the simultaneous gating of smaller conductance states of the channel (in this case, four; Fig. 3A). Subconductance states of the ATP currents of purified human epithelial CFTR were observed (3 of 3 experiments) as indicated above, for currents driven by 100 mM Tris-ATP. At least four identical current levels could be recognized, although the channel gated as a complex of the four levels. Expanded tracings indicated that channel levels “crept up” (Fig. 3B) from seemingly even smaller channel currents, which, as indicated in the third tracing, were consistent with a conductance of 4.5–5.6 pS (see dashed lines).

Effect of low ATP on the Cl⁻ conductance of purified CFTR. To further determine the role of Cl⁻ in the single-channel permeability to ATP, experiments were also conducted in the presence of low cis (5–10 mM) MgATP and high N-methyl-d-glucamine chloride (300 mM) in the trans compartment (Fig. 4A). Both compartments also contained 10 mM MOPS, pH 7.4, and the cis compartment contained 200 nM PKA. Ion channel activity was observed with unitary conductances of 22.2 ± 1.87 pS (n = 11) and 54.9 ± 3.19 pS (n = 20; Fig. 4B). Interestingly, ion channel activity was also observed at positive potentials, indicating a sizable permeability to 10 mM MgATP (identical results were obtained either in the absence of cis MOPS or by its replacement with HEPES). The Eᵥ values of these two channel conductances were highly similar, −15.0 ± 3.54 mV (n = 11) and −18.4 ± 1.0 mV (n = 20, P < 0.3), indicating a Pᵥ/PCl on the order of 9.1–12.9, as obtained when fitting the data with the GHK equation (Fig. 4B). Under similar conditions, a higher conductance state of CFTR, 511 ± 58.1 pS (n = 7), was also observed, albeit rarely, with similar Eᵥ and Pᵥ/PCl of −15.6 mV and 12.6, respectively. In the presence of 300 mM cis Cl⁻ and only 6 mM trans MgATP, with the ionic gradients thus reversed, the single-channel conductance of purified CFTR was 26.6 ± 3.50 pS (n = 16), with an Eᵥ of 16.2 ± 3.98 mV (n = 16) and a Pᵥ/PCl of 17. The data suggest that CFTR may be highly sensitive to external ATP, a phenomenon that will require further examination.

ATP channel activity in the absence of Cl⁻. Single ATP channel activity of CFTR was also determined in the complete absence of Cl⁻ (Fig. 5). With cis 10 mM MgATP and trans 100 mM MgATP (and similar buffer conditions as before), ATP channel currents were observed (Fig. 5, A and B), with single-channel conductance of 32.4 ± 3.10 pS (n = 33; Fig. 5C). Addition of cis Cl⁻, however, reduced the conductance of the ATP channel currents (Fig. 5D). Occasionally, a channel conductance of 235 ± 40.8 pS (n = 5) was also observed. The Eᵥ ranged from −10.5 ± 2.30 mV (n = 33) to −6.3 ± 1.4 mV (n = 5), which was statistically different from the theoretical Eᵥ for MgATP2⁻. (RT/zF)·ln[(ATP)₅₀/
[ATP]_{trans} = 29.1 mV (where [ATP]_{cis} and [ATP]_{trans} are the cis and trans concentrations of ATP, respectively; see Fig. 5C, bottom). Thus it is possible either that ATP\(^{4-}\) (theoretical \(E_r = 14 \text{ mV}\)) makes a contribution to the ionic conductance of CFTR in the presence of MgATP or that CFTR is also permeable to Mg\(^{2+}\). In either case, the CFTR preparation was permeable to ATP, which was further determined by replacing ATP salts. In the presence of trans 100 mM Tris-ATP (cis 10 mM MgATP), the single ATP channel conductance was 38.3 ± 6.1 pS (\(n = 13\)), similar to that seen in the presence of MgATP. The \(E_r\) shifted, however, to −52.6 ± 14.0 mV (\(n = 13\)), consistent with a strong difference in the permeabilities of both free and Mg\(^{2+}\)-complexed ATP. These findings indicate that, although CFTR is capable of Cl\(^{-}\) permeation, its ability to preferentially move Cl\(^{-}\) or ATP may depend not only on the driving force for the intracellular nucleotide ATP but also on the ATP species, the \(P_{ATP}/P_{Cl}\), and the intracellular Cl\(^{-}\) concentration.
tions (5 mM ATP and 30 mM Cl\(^-\)), ATP transport may be favored, however, as the electrochemical gradient for the multivalent ATP will be 1,000-fold higher than that for Cl\(^-\) (18, 22).

**DISCUSSION**

The expression of CFTR has been recently associated with the otherwise unapparent permeation of ATP. This phenomenon has been observed by the cAMP-induced release of ATP from CFTR-expressing cells under physiological conditions (18), as well as by electrophysiological studies under highly nonphysiological conditions (22), which determined, nevertheless, the channel-like nature of the PKA-induced ATP movement at the single-molecule level (17, 22, 23). This is in agreement with our observation that cAMP activation is associated with extrusion of ATP in wild-type (18) but not in Δ508 CFTR-expressing cells (8). The molecular steps associated with ATP movement in CFTR-expressing cells, however, have not been unequivocally determined. Although the simplest possible explanation for these findings is to implicate CFTR itself as the channel responsible for the ATP-permeable pathway, direct evidence to support this claim is still lacking.

ATP-permeable channels in CFTR-expressing cells may be a reflection of endogenous functional channel proteins that are regulated by, but are distinct from, CFTR. For example, expression of both mammalian (1) and *Drosophila melanogaster* (5) P-glycoproteins, CFTR congeners of the ATP-binding cassette (ABC) family of transport proteins, has been associated with the expression of ATP channel activity, thus raising the possibility that transport proteins other than CFTR are present in certain preparations. At least one recent report demonstrated an osmotically stimulated electrodiffusional ATP movement in human hepatoma cells seemingly devoid of CFTR (28). A direct role of CFTR in ATP movement has also been questioned by recent reports that failed to detect ATP-permeable channel activity in cells or preparations containing CFTR (13, 21). However, these studies also failed to detect any other ATP channel activity, which is inconsistent with the ATP release associated with CFTR expression (18, 23). This raises the possibility that the presence of putative ATP-permeable pathways in CFTR-expressing preparations may largely depend on the cellular (tissue) model under study and/or the nature of the ATP complexes driven. Alternate possibilities, however, may also entail more critical experimental conditions for determining ATP movement than previously expected. A previous study that failed to detect ATP release from CFTR-expressing cells (27), for example, may have simply...
Fig. 5. Electrodiffusional ATP movement through purified CFTR in absence of Cl\(^-\). A: purified CFTR, incorporated from cis chamber, was reconstituted into a lipid bilayer chamber containing, on cis side, 10 mM MOPS, pH 7.4, 10 mM MgATP, and 75 nM PKA and, on trans side, 100 mM MgATP, with same buffer composition. \(V_h\) were driven from trans chamber and kept at 150 mV until channel activation was evident. Channel activity was also present at positive \(V_h\), thus indicating ATP currents from 10 mM MgATP. Tracing at bottom left shows expansion of segment marked with a bold line in tracing at top. Histogram on bottom right corresponds to expanded section. Tracings are representative of 16 of 25 experiments.

B: purified CFTR single ATP channels often displayed subconductance states (compare a and b, middle and bottom). N/div indicates the number of 128-sample point bins per division. C, top left: I-V relationships of single-channel currents in asymmetrical MgATP. Two distinct main conductance states were observed under this condition. Solid lines, fits of experimental data to GHK equation. Dashed lines, best linear fits to experimental points with slopes as indicated. C, top right: replacement of trans MgATP with an equimolar concentration of Tris-ATP shifted \(E_r\) but not value of single-channel conductance, indicating that both free and Mg\(^{2+}\)-complexed ATP may have different permeabilities (7 of 7 experiments). C, bottom: I-V relationship is an expansion of C, top left, to more clearly indicate predicted \(E_r\), for \(z = -2\) (right), \(z = -3\) (middle), and \(z = -4\) (left) obtained from fitting with GHK equation of experimental data using asymmetrical MgATP (10 vs. 100 mM) for larger (235 pS) and smaller (32.4 pS) conductances. D: in presence of 10 mM cis MgATP and 100 mM trans MgATP, further addition of cis NMGCl (50 mM) reduced single-channel conductance. Change is shown for 2 channels (top and middle) whose main conductance state was defined as current amplitude at which channel closed (bottom). Single-channel opening (bottom left, a) decreased its main conductance state from 25 pS to 11.7 pS (bottom right, b) after addition of cis Cl\(^-\). Data are representative of 2 experiments.
failed to meet the technological parameters suitable for detecting the expected amounts of ATP released to the extracellular milieu (18). Furthermore, the possibility that under certain conditions, electroneutral ATP complexes are driven through channel structures cannot yet be ruled out.

In this report, we revisited the question as to whether CFTR is responsible for ATP movement. Reconstituted CFTR displayed ATP channel activity either in the absence or presence of Cl\(^-\). Several (including large) conductance states were also observed (see summary of results in Table 2), in agreement with our original in vivo observations (9, 22). In those studies, both a 5-pS and a 50-pS ATP channel were observed. At least two other groups have observed single-channel ATP currents associated with CFTR expression with a similar "low" (5-pS) conductance (16, 17, 23). The issue of the single ATP channel conductance of CFTR may be confounded by the observation that both Cl\(^-\) and ATP modify each other’s movement, thus changing the single-channel conductance of the ions in either direction (22). In the presence of extracellular 100 mM MgATP and 140 mM intracellular Cl\(^-\), the single ATP channel conductances rectified with a limiting conductance of 24.8 pS (Fig. 9 of Ref. 22), which is different from the observed single-channel conductance for either ion alone (22). Furthermore, a recent study also detected Cl\(^-\) channel rectification by CFTR, which is a striking departure from the established paradigm of CFTR behavior (15). This report also summarizes previous data from the literature indicating that single-channel conductances from 7 to 13 pS have been previously attributed to CFTR (Ref. 15, see references within).

In the present report, addition of cis Cl\(^-\) (N-methyl-o-glucamine chloride, 50 mM) in the presence of 10 mM MgATP reduced the CFTR single-channel conductance from 25 to 11.7 pS (Fig. 5D), a condition similar to previously reported values for CFTR-associated Cl\(^-\) movement (13). This finding further suggests that both Cl\(^-\) and ATP may compete with each other for the conductance pore of the channel, as was originally reported in intact cells (22).

Reconstituted CFTR ATP channel activity was readily inhibited by the anti R-domain CFTR antibody MAb 13-1 (Genzyme), in agreement with previous reports in intact cells (19). The possibility that a functional CFTR is required to regulate ATP channels other than CFTR, however, cannot be easily ruled out, since, as indicated above, recent studies failed to detect any ATP-permeable channel activity in preparations containing CFTR (13, 21). At least one such study was conducted with a purified CFTR preparation similar to the one presented in this report (13). Thus a likely possibility is that activated CFTR, which is indeed permeable to Cl\(^-\), may behave as an ATP-selective ion channel only under certain circumstances (see below). It should also be noted that these reports did not exactly mimic our present or previous studies. We have found that PKA activation of CFTR, for example, was readily observed (15 of 18 experiments) in experiments tested with daily fresh batches of PKA. However, under certain experimental conditions, namely the use of “older” PKA (prepared >24 h before the experiments), CFTR activation under Cl\(^-\)-free conditions also required the presence of sustained holding potentials (between +100 and +150 mV; 41 of 82 experiments). This finding may actually explain previous failed attempts to obtain single ATP channel currents from reconstituted CFTR in which PKA was absent (21) and further suggests the possibility that adjacent structures other than CFTR may also be required for proper channel activation in vivo (19).

Although the reason for the required voltage activation of CFTR in the presence of PKA is as yet unknown, it is not unprecedented for ATP transport by other ABC transporters. Concerning the electrodifusional ATP movement by the murine mdr1 gene product, P-glycoprotein (1), for example, it was observed that a double mutation of this channel protein in either nucleotide-binding domain rendered the mutated P-glycoprotein completely unable to spontaneously release cellular ATP. However, voltage activation reestablished ATP transport and, interestingly, Cl\(^-\) channel activity associated with this protein as well (1). Similar findings have also been recently observed with the D. melanogaster mdr gene products Mdr65 and Mdr49 P-glycoprotein homologues, which were also voltage activated (5). Although it is unlikely that this activation process has any physiological significance, it raises the interesting possibility that other relevant intracellular factors that are absent in the purified CFTR preparation may be required for a proper PKA activation of CFTR. Under no circumstances, however, did voltage stimulation induce any ATP-permeable channel activity in the absence of PKA.

The present data also dispel a common misconception about the size of ATP as it is transported through this channel molecule. In solution, ATP takes various conformations by interacting with counterions other than Mg\(^{2+}\) (11). However, only one conformation has been observed for the MgATP\(^{2+}\) complex, the relevant moiety associated with molecules that bind and/or hydrolyze ATP (4, 11). The average planar diameter of the MgATP complex

Table 2. Most frequent single-channel conductances

<table>
<thead>
<tr>
<th>cis/trans Concentrations, mM</th>
<th>n</th>
<th>Conductance, pS</th>
<th>E(_r), mV</th>
<th>P(_{\text{ATP/Cl}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymmetrical Cl(^-)/ATP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 Cl(^-)/100 Tris-ATP</td>
<td>10</td>
<td>8.6±0.2</td>
<td>2.2±1.8</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>34.3±0.3</td>
<td>0.4±0.3</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.5±5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300 Cl(^-)/6 MgATP</td>
<td>16</td>
<td>26.6±3.5</td>
<td>16.2±4.0</td>
<td>17.0</td>
</tr>
<tr>
<td>5–10 MgATP/300 Cl(^-)</td>
<td>11</td>
<td>22.2±1.9</td>
<td>13.9±3.5</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>54.9±3.2</td>
<td>18.4±1.0</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>511±36.1</td>
<td>13.6±5.7</td>
<td>12.6</td>
</tr>
<tr>
<td>Asymmetrical ATP salts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 MgATP/100 MgATP</td>
<td>33</td>
<td>32.4±3.1</td>
<td>10.5±2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>235±40.8</td>
<td>6.3±1.4</td>
<td></td>
</tr>
<tr>
<td>10 MgATP/10 Tris-ATP</td>
<td>13</td>
<td>38.3±6.1</td>
<td>52.6±14.0</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE for n experiments. Most frequent single-channel conductances in presence of various concentrations of asymmetrical Cl\(^-\)/ATP are shown. ATP over Cl\(^-\) perm-selectivity ratios (P\(_{\text{ATP/Cl}}\)) were calculated from best fitting of Goldman-Hodgkin-Katz equation under experimental conditions indicated. Also shown are most frequent single-channel conductances of ATP single-channel currents in asymmetrical ATP salts and/or concentrations. *Subconductance states. E\(_r\), reversal potential.
under these conditions is <5 Å (see Fig. 6), at least similar to, if not smaller than, the expected diameter of I⁻, a CFTR-permeable anion (for details, see Fig. 6). Thus ATP “size” is not a constraint to movement through CFTR, provided that it is either free or complexed to Mg²⁺. Counterion-ATP complexes of larger sizes, however, may reflect different interactions with CFTR. A recent paper (13), reporting that purified CFTR was tested and failed to detect ATP transport, suggested that the K₂ATP salt used in that study actually blocked Cl⁻ movement through CFTR. Because K⁺ is almost nine times larger than Mg²⁺, the larger K₂ATP moiety is likely to be a blocking agent of CFTR channel activity. This is in agreement with an expected, undetectable, 0.14-pS K₂ATP single-channel conductance (assuming a monovalent salt as the minimum size) calculated by extrapolating the single-channel conductance of 26.2 pS obtained in the present study, as a function of the counterionic radius of the MgATP complex.

Reconstituted CFTR mediated the electrodiffusional movement of both Cl⁻ and ATP and displayed several
conductance states, which may be related to "clustering" of CFTR in complexes that have the same permeability properties. Each ion seems to modify the permeability properties of the other, which may be due to either competition for one another at the conductance pore of the channel or the possibility that both ions have distinct, but mutually interactive, permeable pathways. This is a phenomenon that will require further investigation. Interestingly, however, both the cis concentration of Cl− and the trans concentration of ATP modified the single-channel conductance of CFTR.

In conclusion, the present data are consistent with the most likely, but not exclusive, possibility that CFTR permeates ATP. This is in close agreement with previous reports indicating that wild-type CFTR (9, 17, 22, 23), but not Δ508 CFTR (8), may be involved in ATP movement in vivo. "Contaminant" channels that are permeable to ATP but are distinct from CFTR cannot be ruled out, although the finding that preparations with different degrees of purification of CFTR, including the purified protein, show functional characteristics similar if not identical to those observed in vivo strongly suggest a direct role of this channel protein in ATP movement. Additional studies, including mutational analysis of this channel protein, will be required, however, to further assess the relevance of the CFTR-mediated ATP transport in the context of Cl− vs. ATP transport and, most importantly, in the onset and reversal of the CF phenotype.

We gratefully acknowledge Dr. Yi Wang for assistance in the analysis of the structural models of ATP.

Address for reprint requests: H. F. Cantillón, Renal Unit, Massachusetts General Hospital East, 149 13th St., Charlestown, MA 02129.

Received 16 May 1997; accepted in final form 26 November 1997.

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