Cystic Fibrosis Transmembrane Conductance Regulator and Adenosine Triphosphate

The observations by Abraham *et al.* (*1*), that P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR) are associated with adenosine triphosphate (ATP) movement across the plasma membrane, have been supported by other studies using patch clamp and bulk ATP measurements of systems expressing CFTR (*2*, *3*). On the other hand, Reddy *et al.* (*4*) and Li *et al.* (*5*) did not detect ATP channel activity with electrophysiological methods or with radioactive ATP in reconstituted vesicles that contain CFTR. They state that CFTR does not conduct ATP, but that it might act as a regulator of an associated channel or transport system for ATP, a possibility also raised by Al-Awqati (*6*) and Higgins (*7*).

Although there is conjecture that CFTR can form a complex with channels for anions (*3*) and cations (*8*), it is not excluded that CFTR and other ABC proteins are themselves directly involved in ATP movement. The resolution of this issue is hampered by disagreement about the mechanism of the ATP movement associated with ABC transporters: whether it is electrodiffusional through a channel or by facilitated transport. A further complication is that the form of ATP that moves across the membrane may vary, depending on the experimental conditions, between the fully charged ATP^{-4} anion and the uncharged $ATP^{-3}(Mg^{+2}{\cdot}L^{+})$ molecule, where L^{+} is a cotransported solute.

Both ATP and adenosine diphosphate (ADP) are capable of forming complexes with P-glycoprotein substrates in cell-free

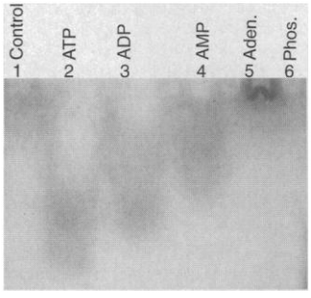

Fig. 1. Comparison of the effect of various adenylates on the movement of a homogeneously doxorubicin (200 μg/ml) impregnated polyacrylamide gel. The gel was exposed to 100 volts for 30 min. Well 1 was loaded with 30 μl of tris-glycine buffer (control). Loading was 30 μl of 10 mM ATP in well 2, 30 μl of 10 mM ADP in well 3, 30 μl of 10 mM adenosine monophosphate (AMP) in well 4, 30 μl of 10 mM adenosine in well 5, and 30 μl of 10 mM phosphate in well 6. All the doxorubicin traveled upward toward the cathode in lane 1. Flow of doxorubicin toward the anode was seen in the ATP loaded lane and to a lesser extent in the ADP loaded lane. Adenosine and phosphate had doxorubicin mobilities similar to control. Experiments were performed at pH = 7, 20°C. The experiments demonstrate the potential of ATP-dependent cotransport to explain the removal of cationic and zwitterionic drugs mediated by P-glycoprotein and MRP. This mechanism, based on electrostatic coupling, requires that both ATP and drug are transported through P-glycoprotein.

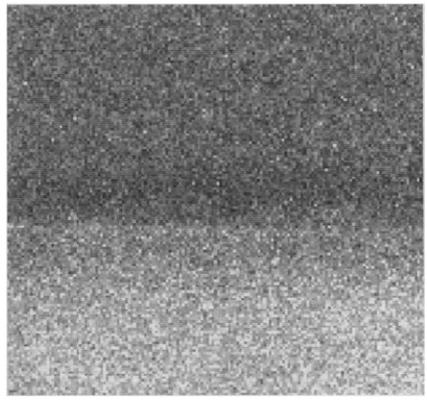

Fig. 2. Comparison between steady-state light output generated by luciferin/luciferase-treated T84 cells, colon carcinoma cells overexpressing CFTR, compared with SW620, colon carcinoma cells with low detectable expression of P-glycoprotein and no detectable CFTR. Light detection was performed using a high-speed CCD camera (Princeton Instruments LN/CCD-1024TK[B]) mounted on an Olympus inverted microscope. Cells were plated in 96 well Wallac Inc. (LB96PMP) plates. Each 6-mm diameter well was partitioned using stainless steel dividers. The images are 3 by 3 mm and 1000 cells were plated on each side of the divider in a volume of 150 μl. Fifty-microliter aliquots of luciferin/Luciferase Assay Mix (Sigma FL-AAM, Lot 45H8000) were added to both sides of the well. The CFTR overexpressing T84 cells have greater light output during a 10-min exposure. Light output is a power function of ATP concentration and the assay is specific for ATP. Other nucleotide phosphates do not produce light. Experiments were also performed as with SW620/AD300 cells, overexpressing P-glycoprotein, and control SW620 cells (data not shown); 10^4 cells of each cell line were plated in 150 μl on opposite sides of the divider. The P-glycoprotein overexpressing SW620/AD300 cells, like the CFTR overexpressing cells, have greater light output compared to the control population.

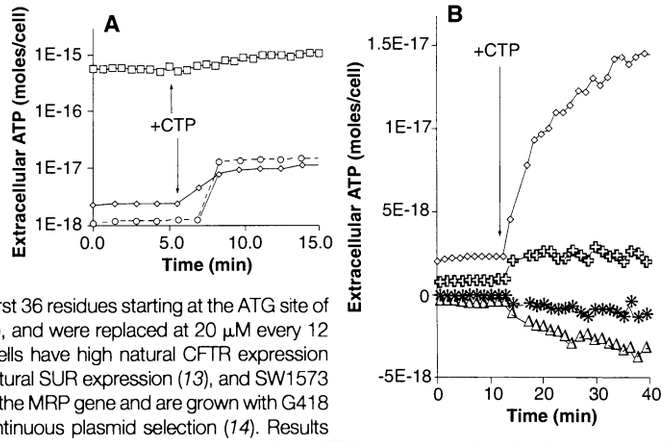

Fig. 3. (**A**) Steady-state extracellular ATP and extracellular ATP accumulation after ecto-ATPase inhibition [by added 0.5 mM cytidine triphosphate (CTP); Pharmacia Biotech] were measured from cells with tandem 18-mer sense and antisense oligonucleotides. The tandem oligonucleotides were directed at the first 36 residues starting at the ATG site of the relevant ABC gene (*14*), and were replaced at 20 μM every 12 hours for 36 hours. T84 cells have high natural CFTR expression (*14*), RIN cells have high natural SUR expression (*13*), and SW1573 cells were transfected with the MRP gene and are grown with G418 containing medium for continuous plasmid selection (*14*). Results are expressed as the difference between the control extracellular ATP (sense-treated) and antisense-treated extracellular ATP, normalized per cell. Constant volumes of media and constant cell numbers were used in all experiments. Antisense to the specific ABC protein reduced both steady-state extracellular ATP and accumulation of extracellular ATP following CTP inhibition of ecto-ATPase. Extracellular ATP release was measured using ATP Assay Mix (Sigma FL-AAM) and a Wallac Inc. LB96P luminometer. There were 2000 cells per well. Experiments were performed twice with 12 replicates per experiment. T84 cells = CFTR overexpression (ATP release from CFTR sense-treated cells minus ATP release from CFTR antisense-treated cells) (□); RIN cells = SUR overexpression (ATP release from SUR sense-treated cells minus ATP release from SUR antisense-treated cells) (◇); MRP transfected cells (SW-1573) (ATP release from MRP sense-treated cells minus ATP release from MRP antisense-treated cells) (○). (**B**) Steady-state extracellular ATP, and its accumulation after CTP are measured as in (A). Steady-state extracellular ATP of RIN cells is decreased maximally by SUR antisense, and differential extracellular ATP is over 100-fold greater for the SUR sense–antisense-treatment pair as compared to other oligonucleotide pairs of ABC genes not over-expressed by RIN cells, including P-glycoprotein, MRP, and CFTR. Experiments were performed twice with 12 replicates per experiment. RIN cells = SUR overexpression (ATP release SUR sense-treated cells minus ATP release SUR antisense-treated cells) (◇); RIN cells = SUR overexpression (ATP release MRP sense-treated cells minus ATP release MRP antisense-treated cells) (+); RIN cells = SUR overexpression (ATP release MDR sense-treated cells minus ATP release MDR antisense-treated cells) (*); RIN cells = SUR overexpression (ATP release CFTR sense-treated cells minus ATP release CFTR antisense-treated cells (△).

electrochemical models (Fig. 1), where positively charged doxorubicin moves toward the positive electrode in the presence of ATP and ADP. In related electrophoresis experiments, camptothecin (which is not a substrate for P-glycoprotein) is unaffected by ATP, while positively charged but structurally similar topotecan (a P-glycoprotein substrate) is cotransported with ATP in the electric field (9). Cotransport of ATP with cationic molecules can result in nearly electroneutral ATP efflux across membranes. Net electroneutrality would also occur if CFTR functions as an anion exchanger (for example, chloride-ATP). Under these circumstances, the ability to detect ATP currents in patch clamp studies would be more difficult than measurement of bulk ATP movement.

The structural similarities of P-glycoprotein and CFTR suggest that both proteins are involved in ATP transport in the same fashion. This view is supported by the observation (Fig. 2) that cells overexpressing CFTR or P-glycoprotein are associated with increased release of ATP. Furthermore, exposure of cells overexpressing CFTR, sulfonylurea receptor (SUR), or multidrug resistance–associated protein (MRP) to antisense oligonucleotides specific for the respective ABC protein (*10*) results in reduced bulk ATP exit as measured by bioluminescence (Fig. 3). These observations indicate that several ABC proteins, all of which share similar structures (*7*), are associated with ATP transport.

There are at least two limiting explanations for the difference in the results on ATP movement by CFTR, and other ABC proteins, in the literature. One is that CFTR is not itself an ATP transporter-channel and that ATP movement in cells with different ABC transporters is mediated by an associated ATP transporter-channel, as implied by Reddy *et al.* (*4*) and Li *et al.* (*5*). The other and perhaps more likely explanation is that CFTR is an ATP transporter-channel and that detection of ATP movement may be problematic because of small or negligible currents or altered protein conformation (*11*) attending reconstitution into liposomes and the absence of regulatory protein interactions (*12*).

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Response: Prompted by reports that CFTR is an ATP ion channel (*1*, *2*), we conducted experiments (*3*, *4*) to detect CFTR-mediated ATP currents using bilayer studies, patch clamp recordings, or transepithelial conductance measurements. We did not detect any evidence for ATP conductance through CFTR in four different preparations using five different recording methods (*3*). A subsequent paper supports these findings (*5*). Abraham *et al.* propose that expression of CFTR might be associated with increased ATP release from cells by a mechanism that would be invisible to patch clamp recordings, such as electroneutral transport. Thus, Abraham *et al.* also appear to conclude that CFTR is not an ATP channel, but raise another issue of whether ATP release is influenced in any way by CFTR expression. The results in the following response address that possibility.

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Response: Studies by Reddy *et al.* (*1*), Grygorczyk *et al.* (*2*), and Li *et al.* (*3*) indicate—contrary to the previous reports by Reisin *et al.* (*4*), Schwiebert *et al.* (*5*), and Pasyk and Foskett (*6*)—that CFTR does not conduct ATP at rates that can be measured electrophysiologically ($>10^5$ s^{-1}). Nevertheless, the results of the former group did not exclude alternative mechanisms, such as a low rate of ATP conduction through CFTR, electroneutral ATP transport, or CFTR-dependent regulation of ATP flux through other pathways. To test these possibilities, two of which are described in the comment by Abraham *et al.*, we studied ATP efflux using luciferin-luciferase luminometry, a method that is potentially 10^4-fold more sensitive than are electrical methods for detecting ATP flux and should measure charged and uncharged forms of ATP.

We found that T84 cells, which express high levels of endogenous CFTR, did not release ATP at a detectable basal rate, nor is release stimulated by adenosine 3′,5′-monophosphate (cAMP) (*7*). Cells were studied on a rotating platform so that secreted ATP could escape hydrolysis by ecto-ATPases at the cell surface. Because ATP transients might be missed even at our shortest sampling interval (2 min), we also recorded luciferase luminescence online; that is, with cells in the luminometer cuvette. Addition of CTP (to block ecto-ATPases) increased luminescence (Fig. 1, traces labeled "a") with a time course closely resembling that shown in figure 3 of the comment by Abraham *et al.*; however, in our hands this result was similar regardless of cell type and was not correlated with expression of CFTR or MDR. Moreover, the responses were a result of ATP contamination in all the commercially available lots of CTP tested (4 to

400 nM; average contamination 0.001%), and disappeared if CTP solutions were depleted of ATP by preincubation with luciferase (Fig. 1, traces labeled "b").

Abraham *et al.* describe large differences in the ATP concentration of solutions bathing T84 cells that had been treated with sense as opposed to antisense oligonucleotides to CFTR even before addition of CTP (about 6 nM, from figure 3A of the comment), although actual ATP concentrations are not given. This "difference" measurement is much larger than the absolute (unsubtracted) ATP amounts in our experiments, although the latter would, if anything, be expected to overestimate ATP release. The high ATP concentrations evident in figure 3 of the comment by Abraham *et al.* would require release of at least 5 to 10% of the total cellular ATP content. Such release could be induced by cell lysis or subtle differences in the handling of sense and antisense cells (see below). The inability to measure ABC transporter-dependent ATP release in our experiments cannot be attributed to inadequate sensitivity; our signal-to-noise ratio should have been improved by 10^2- to 10^3-fold, as compared with that found by Abraham *et al.*, because we used many more cells in the same reaction volume.

Massive ATP release was triggered, however, by transferring the glass coverslip with cells into the luminometer cuvette, generating a luminescence peak corresponding to 4.5 nM ATP. This represents release during transfer, because ATP was not detected in the medium from which cells were removed. Concentrations of 2 nM were produced when solution was injected through the inlet port or added directly to the cuvette using a micropipetter. Thus, injecting cAMP caused transient elevation of ATP (Fig. 2A), and mock injection of isotonic NaCl solution gave a similar response (Fig. 2B). ATP release did not occur when the cover slip was oriented so that the cells faced away from the injection port and cAMP (or NaCl solution) was gently introduced into the cuvette without turbulence (Fig. 2C). We recently found that similar ATP release can be induced by mechanical stretching when cells are grown on a flexible substrate, and proposed that mechanically induced release may be a physiologically relevant mechanism of ATP secretion (8).

In summary, we could find no evidence using luciferase luminometry that ATP release is related to CFTR or MDR expression, although ATP is released by very slight mechanical stimuli. Extracellular ATP released by physical perturbations may interact with purinergic receptors and play an important role in regulating epithelial transport (9) and cell volume (10).

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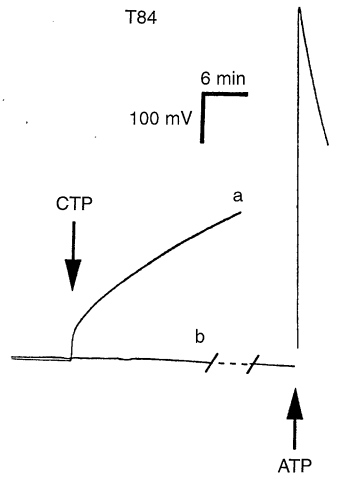

Fig. 1. Effect of CTP (added to inhibit ecto-ATPase activity) on extracellular ATP concentration, measured with cells in the luminometer cuvette. Luciferase luminescence was monitored with T84 cells expressing endogenous CFTR (data shown), baby hamster kidney (BHK) cells stably expressing high levels of wild-type CFTR (*11, 12*), Chinese hamster ovary (CHO) cells expressing multidrug resistance protein (MDR), and control (untransfected) CHO cells. Data was similar for all four assays (data shown only for the first). Trace labeled "a": CTP (0.5 mM) significantly increased luminescence (compare with figure 3 of the comment by Abraham *et al.*). Luminescence elicited by CTP was caused by ATP contamination because it disappeared when CTP was depleted of ATP by pretreatment with luciferase (trace labeled "b"). Addition of 5 nM ATP to the cuvette at the end of each experiment (approximately the subtracted values estimated from figure 3 of the comment by Abraham *et al.*) confirmed that luciferase had retained its full responsiveness to ATP. The five different lots of CTP from Pharmacia and Sigma examined had ATP contamination between 4 to 400 nM/1 mM CTP, well within the manufacturer's specifications (98 to 99.9% purity). Standard hexokinase + glucose pretreatment did not reduce ATP contamination significantly, perhaps because the concentrations are at least 10^4-fold below the K_d of hexokinase for ATP. Pre-incubating solutions (1 mM final CTP concentration) with luciferase overnight at 4°C followed by 2 hours at 20°C reduced ATP contamination to less than 0.05 nM. Cells were grown on glass cover slips of 6 by 8 mm as described (*8*). It was necessary to allow them to equilibrate for 30 to 60 min because transfering cells to the cuvette caused a massive release of ATP. This equilibration period permitted extracellular ATP to decline below the detection limit of the assay (0.02 to 0.05 nM) before experiments were started.

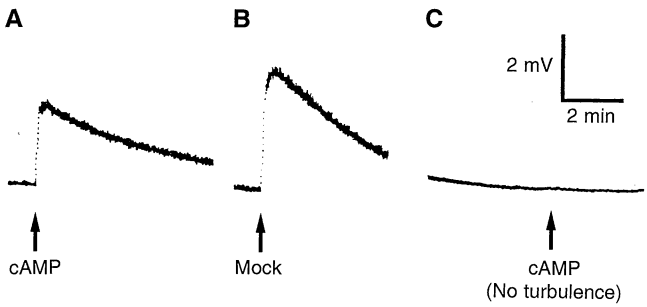

Fig. 2. ATP release is acutely sensitive to solution turbulence. (**A** and **B**) ATP transients induced by standard injection through the injection port, using a Hamilton syringe, of cAMP (to stimulate CFTR) and mock injection of NaCl solution gave similar responses, with peak luminescence corresponding to 0.20 to 0.23 nM ATP above the baseline of 0.02 nM ATP. (**C**) Gentle delivery of cAMP mixture to the cuvette via polyethylene tubing inserted behind the cover slip to avoid turbulence did not produce detectable ATP release.

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7. Stimulation of CFTR-expressing cells (T84) by cAMP does not elevate extracellular ATP concentration. Three monolayers of T84 cells were exposed to vehicle and another three monolayers to cAMP cocktail (500 μM 8-Br-cAMP, 100 μM IBMX, 10 μM forskolin). Samples were collected from each monolayer before addition (0 min) and at 10 and 60 min after addition. For all samples, luminescence remained at background levels despite high cellular ATP content (~5 to 7 mM), which was determined by lysis at the end of each experiment. Similar results were obtained with other cell lines: Calu-3, 9HTEo$^-$, ΣCFTE29o$^-$, CHO (control cells transfected with pNUT vector alone and a line stably overexpressing CFTR) and NIH 3T3 (*8*). Cell monolayers (9.4 cm^2) were grown in six-well plates as described (*8*). Experiments were performed after three washes with NaCl solution and 2 hours pre-equilibration at 37°C, using a platform shaker to reduce the unstirred layer.
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