Antibiotic accumulation and membrane trafficking in cystic fibrosis cells

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Cystic fibrosis (CF) results from mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) which is a regulated chloride channel. The ΔF508 mutation prevents the post-translational glycosylation and membrane insertion of the protein. Severe disease follows, with the formation of a viscous mucus and subsequent chronic bacterial infection of the lungs, necessitating frequent, and often long, periods of antibiotic treatment. The pharmacokinetics of antibiotics in CF patients are abnormal, with lower blood serum levels and higher clearance rates which have never been satisfactorily explained. We found that accumulation of gentamicin in nasal polyp tissue non-CF cells was subject to regulation by the effectors and inhibitors of CFTR function; regulation was lost in ΔF508 CF cells and accumulation was more than doubled because of the inhibition of exocytosis.

Introduction

Since the discovery and sequencing of the cystic fibrosis (CF) gene in 1989, over 629 mutations have been reported; the disease, though commonest in Caucasians, has also been described in non-Caucasians. Knowledge of the related physiological consequences of these mutations has enabled classification of CF genotypes into five classes. Class I comprises nonsense, frameshift and splice mutations that severely limit or prevent synthesis of the CF transmembrane conductance regulator (CFTR) protein. In class II, full post-translational processing of CFTR is blocked; this class includes the most common CF mutation, ΔF508. Class III mutations yield non-functional channels that are unable to open when stimulated by cyclic AMP (cAMP), class IV mutations alter channel conductance or ion-selectivity, while class V mutations reduce the amount of functional CFTR produced. Mutations in classes I–III have severe disease consequences, while those in classes IV and V are less severe.

In normal cells in which the gene is operational, the CFTR protein is located in the apical cell membrane and is a low conductance (4–7 pS) chloride channel whose function is essential for maintaining airway integrity. Recently it has also been shown to be a negative regulator of the epithelial Na+ channel, so acting as a switch that balances the processes of Na+ absorption and Cl− secretion. The net results of the altered ion gradients that occur in CF are the evolution of viscous mucus, impaired ciliary clearance and subsequent chronic bacterial infection of the lungs.

In the ΔF508 class II mutation, the modified protein fails to undergo post-translational glycosylation. Post-translational processing is closely linked with the efficient delivery of ‘finished’ proteins from the endoplasmic reticulum to the membrane site and, at 37°C, ΔF508 protein is destroyed before it can be established in the membrane. Processing is itself temperature-sensitive and, at the lower temperature of 26°C, protein does stabilize in the membrane. Moreover, this mutant protein, when present in the membrane, does act as a chloride channel and appears to be regulated.

Regulation of CFTR function occurs at two (or more) levels. cAMP activates protein kinase A (PKA) which then phosphorylates the R domain of CFTR by transfer of PO43− from ATP. There are also two nucleotide-binding domains (NBDs) and phosphorylated CFTR actively binds ATP. The hydrolysis of bound ATP is a necessary energy-consuming step in the opening of the CFTR channel and Cl− conductance.

The extensive colonization of the CF lung by bacteria necessitates frequent and often long periods of antibiotic treatment by injection or, latterly, by direct inhalation. In CF patients the pharmacokinetics of antibiotics have long been known to be abnormal; lower blood concentrations and higher clearance rates have been found for many
antibiotics, including dicloxacillin,22 epinephrin and cepha-lizin,23 methicillin,24 gentamicin,25 tobramycin26–28 and amikacin.29,30 The necessity for higher dosing levels carries the risk of increased drug toxicity.

In this report we confirm the link between accumulation of antibiotics and the function of the CFTR channel and show that, in CF cells, gentamicin is transported in vesicles whose recycling to the outer cell membrane is blocked by the loss of CFTR channel function.

Materials and methods

Tissue and culture methods

Nasal polyp and lung tissue was obtained at biopsy from homozygous ΔF508 CF patients and from non-CF subjects and cultured directly as described previously.31 The polyp CF cultures were from a 3.5 year old male (A), a 10 year old female (B) and a 4 year old male (C). The non-CF polyp cultures were from a 47 year old male (W), a 36 year old female (X); a 26 year old female (Y) and a 56 year old female (Z). Experimental procedures for accumulation and efflux determination were as described.31

Antibiotic and protein assays

After treatment, cells were harvested, washed and dis-integrated with Triton X-100 as described31 and the homogenate was assayed for antibiotic by a microbiological method using Microdetection plates (Proteus Molecular Design Ltd, Macclesfield, UK). The limit of sensitivity for gentamicin was 0.25 mg/L, using Bacillus subtilis ACTC 10400 spores. Protein assays were done using a modified Lowry method.32

Activators and inhibitors

To stimulate activation of PKA, a ‘cocktail’ of 10 μM forskolin, 1 mM 3-isobutyl-methyl-xanthine (IBMX) and 200 μM 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphate (8-CPT-cAMP) was used. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7; 100 μM) was used to inhibit PKA and the non-hydrolysable ATP analogue 5'-adenylylimidodiphosphate (AMP-PNP; 5 mM) was used for competition with ATP.

Fluorescence labelling and measurement of accumulation

Fluorescein isothiocyanate (FITC; 1 mg/L) and FITC-gentamicin (10 mg/L; a gift from Abbott Laboratories, Maidenhead, U.K.) were used to measure accumulation and visualize the localization of gentamicin within cells. Laser confocal microscopy on single cells was done on a Bio-Rad MRC 600 with a 25 mW argon laser (pinhole 2 mm, white gain 9.0, black gain 4.91). Accumulated fluorescence in 10,000 cell samples was measured on a Becton Dickinson flow cytometer (FACSCAN) using the program Lyses II v1.2.

Results

A accumulation of antibiotics in CF cells

In an earlier paper23 we reported the first measurements of antibiotic accumulation in CF cells; cloxacillin in ΔF508 cells was about 33% higher than in control cells. We have extended the previous work with gentamicin sulphate (Gm) to three different CF cell lines and now confirm that the mean increase in accumulation after 96 h contact at 37°C was more than twice that in control cells (P < 0.001; Figure 1). We have also shown that Gm accumulation in CF cells was not sensitive to cAMP, but was very sensitive to it in non-CF cells. We further showed that the specific reason for the excess accumulation was the greatly diminished ability of CF cells to efflux Gm, without blockage of the ability to take up gentamicin.31 Similarly increased accumulation was shown for the fluorescent dye 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole. We have now directly tested the converse of cAMP stimulation; i.e. the antagonism of CFTR function which should increase accumulation and cause the inhibition of exocytosis in normal cells.

Inhibition of CFTR function causes antibiotic accumulation

Activated PKA is necessary for the phophorylation and subsequent Cl– channel function of CFTR,5,17,18 so the

Figure 1. The accumulation of gentamicin sulphate by CF ΔF508 cells and non-CF cells after 95 h contact with 400 mg/L gentamicin sulphate in growth medium. A aggregated means ± s.d. for CF cells A (n = 8), B (n = 4) and C (n = 3), and non-CF cells X (n = 8) and Y (n = 7) are shown. P < 0.001 for mean difference. The value for rat fibroblasts (RAT-fib) was extrapolated for equivalent gentamicin concentration from data in Tulkens et al.38
inhibition of PKA should have the opposite effect to the increased availability of cAMP and PKA activation provided by a cocktail containing forskolin, IBMX and 8-CPT-cAMP. When PKA was inhibited by H7, the effect on accumulation of gentamicin in both CF and non-CF cells was dramatic. CF cells accumulated 53% more antibiotic in the presence of H7 than in its absence (P < 0.001), while non-CF cells increased accumulation by 133% over controls (P < 0.001; Figure 2).

When cells were pre-loaded with antibiotic by incubation for 72 h in the presence of gentamicin, washed, and then allowed to efflux antibiotic in the presence of H7, both CF and non-CF cells effluxed only 5% of internal gentamicin after a further 72 h incubation. However, when H7 was omitted from the efflux environment CF cells lost 25% and non-CF cells 52% of the pre-loaded values (P < 0.001; Figure 2). Clearly the inhibition of PKA prevented loss of antibiotic from cells.

Similarly, ATP is required for CFTR function, both for phosphorylation of the R domain and for binding to the NBDs, triggering channel function. The presence of a non-hydrolysable analogue of ATP would, therefore, antagonize CFTR function. Since, initially at least, there would be competition between internal ATP and the incoming analogue, the effect of such an ATP analogue would be similar to that of PKA inhibitors, but reduced in extent. Eventually all binding sites would be blocked and this avenue of ATP consumption would be closed.

When CF cells were incubated with 5 mM AMP-PNP, there was only 10% more gentamicin accumulation than in the absence of AMP-PNP. However, the accumulation in non-CF cells increased by 101%. As in the case of H7, this effect on normal cells is equivalent to the effect of the mutation to ΔF508 since, in the absence of agonists or antagonists, cells with the CF mutation accumulate over twice as much gentamicin as non-mutated cells. It is tempting to conclude that, at the physiological level, the cause is the same, namely inhibition of exocytosis resulting from failure of function of the CFTR. This conclusion was further supported by the results of efflux experiments. Efflux from pre-loaded CF cells in the presence of AMP-PNP was again 5%, and that from non-CF cells was 17%. However, when AMP-PNP was omitted from the efflux environment, effluxed antibiotic was 31% and 50% of pre-loaded values, respectively (Figure 3). The difference in proportion effluxed from non-CF cells compared with CF was significant (P < 0.05).

**CFTR function is linked to membrane trafficking**

In the non-CF colonic epithelial T84 cell line, forskolin and the membrane-permeable cAMP analogue CPT-cAMP both had significant inhibitory effects on endocytic uptake of horseradish peroxidase under conditions that stimulate Cl⁻ secretion. Conversely, forskolin did not inhibit endocytosis in cells of a CF pancreatic epithelial cell line, but transfection of the CFTR gene into this line restored both the cAMP regulated Cl⁻ secretion and the cAMP regulated inhibition of endocytosis. It was further shown that CPT-cAMP greatly increased the amount of internalized biotinylated wheat-germ agglutinin incorporated into the external membrane by exocytosis in cells with functional CFTR. Conversely, neither of these agonists stimulated exocytic recycling in cells with the ΔF508 double mutation.

To confirm the role of membrane trafficking in the variation of gentamicin accumulation recorded in this
study, we treated control cells and CF cells with FITC alone or with FITC–gentamicin, and examined them by confocal microscopy and flow cytometry. Confocal microscopy showed that gentamicin was localized in vesicles within the cells (Figure 4). In non-CF cells optical sections 1.5 µm thick showed fluorescent inclusions more or less evenly distributed throughout all areas of the cell cytoplasm (Figure 4a). However, in CF cells similarly treated, sections of the same thickness showed that the outer regions of the cell cytoplasm contained very few or no fluorescent inclusions, while the number of inclusions and fluorescent intensity increased towards the central areas of the cell (Figure 4b).

Micrographs of whole-cell fluorescence (not shown) also clearly showed that fluorescence accumulated in single cells towards the cell centre and that fluorescence was more intense in CF cells than in non-CF cells. Digitization of whole-cell fluorescence intensity in single cells confirmed that CF cells accumulated more than twice the amount of label than did non-CF cells. This is exactly what would be expected if, in normal cells, FITC–gentamicin was engulfed by endocytosis into vesicles which were then cycled through the cell and eventually reintegrated with the membrane by exocytosis, while in CF cells the inhibition of exocytosis would lead to accumulation of vesicles in the cell centre. To confirm that this excessive accumulation of label (and hence of antibiotic) was a general phenomenon of the whole population, samples of the same cell suspensions were subjected to flow cytometry. The FACSCAN analysis of fluorescent intensity of 10,000-cell samples of each population is given in the Table, along with the data for equivalent cultures of two different cell lines incubated with FITC alone. The mean accumulation of gentamicin in CF(A) cells was 2.6 times greater than that in the non-CF(Z) cell line. The mean accumulation of FITC in CF(B) cells was 1.9 times greater than that in non-CF(W) (Table).

CF cells have high ATP and energy charge levels
The exact role of ATP in the activity and regulation of the CFTR channel is not well understood. Normal physiological levels of ATP (c. 5 mM) are required for efficient function, and reduction to concentrations below this level diminish conductance until it ceases at about 1 mM ATP. ATP is required for the phosphorylation that activates CFTR and there is evidence that at least four sites on the R domain must be phosphorylated. Hydrolysis of ATP is essential in this process, but this PKA-dependent phosphorylation is relatively irreversible so would not be a serious drain on cell energy resources. However, the two NBDs do bind ATP and hydrolysis appears necessary for the conductance of chloride. Such a requirement would involve considerable expenditure of energy since each channel-opening episode depends on ATP hydrolysis, so that in CF cells where conductance is "blocked" (either because the CFTR is not membrane-located or because it is membrane-located but non-functional) or in normal cells where CFTR function is blocked by application of antagonists, ATP would be expected to accumulate to levels higher than those in normally functioning cells. We measured the levels of adenosine nucleotides in CF and non-CF cells and found that the mean energy charge (n = 3) in CF cells was 0.75, as compared with 0.67 for non-CF cells (P <0.05), reflecting the greatly increased level of ATP in CF cells and the higher turnover of energy in non-CF cells. The
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Discussion

Our results are compatible with published findings on the cellular physiology of CF cells. They show that CF cells accumulate over twice as much gentamicin as non-CF cells, and that this results primarily from the inhibition of exocytosis when the CFTR channel is inoperative.

The cAMP stimulation of conductance in non-CF cells decreased accumulation by about 50%, by increasing the exocytic arm of membrane trafficking, but had little effect on accumulation in CF cells or on influx. Conversely, the inhibition of PKA increased accumulation in both CF and non-CF cells, 1.5-fold in the former and 2.3-fold in the latter, because of the reduced efflux by exocytosis. When the non-hydrolysable ATP analogue AMP-PNP was present, the results were similar, but less marked than those for PKA inhibition.

The accumulation of aminoglycosides in rat fibroblast cells has been measured previously. At an external drug concentration of 500 mg/L after 96 h contact the accumulation ratio was 5.2 for gentamicin, 5 for amikacin, and 2 for both streptomycin and kanamycin. Similar calculations using the data from Figure 1 gave mean accumulation ratios for gentamicin in human polyp cells of 5.9 for non-CF cells and 12.9 for CF cells in the absence of agonists or antagonists of CFTR function. The accumulated antibiotic is concentrated in vesicles, visualized by confocal microscopy, located centrally within the cytoplasm in CF cells.

It has been argued that a conformational change in CFTR brought about by NBD binding of ATP could be enough to cause opening of the channel and chloride conductance that occurs in response to the ion gradient and does not, per se, require energy. However, there is good evidence that hydrolysis is essential for chloride conductance, supported by our AMP-PNP data and elevated ATP levels and it is probable that the high energy consumption in the process may be linked to the equally important phenomena of membrane cycling by which the composition of the outer membrane itself, and hence the physiological functions dependent on membrane-located enzymes, is changed. These considerations are important since the energy charge of a cell is itself a regulator and balancing factor between the catabolic and anabolic arms of metabolism in human cells as it is in bacteria. In normally functioning cells, the net accumulation is the difference between the amount of drug endocytosed and that lost by exocytosis or biotransformation. The CF mutation alters the balance between endocytosis and exocytosis, which is normally regulated by cAMP and ATP levels which also regulate channel function. This increased concentration of antibiotic within CF cells may explain the findings (for cloxacillin) of Spino et al. that ‘non-renal clearance in the group with CF was 2.5 times that in controls’, but would contradict their suggestion that ‘the most likely cause is increased biotransformation’. These results also highlight the necessity for further work on the accumulation of antibiotics in cells specifically subject to toxic effects. Such work should be expedited in view of proposals for once-daily high-level aminoglycoside regimes for treatment of CF patients.

Acknowledgements

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Table. Parameters of the distributions (FACSCAN) of fluorescence intensity of 10,000-cell samples of (a) CF(B) and non-CF(W) cell lines incubated for 24 h in medium alone and with 1 mg/L FITC and of (b) CF(A) and non-CF(Z) cultures after 48 h in medium alone and with 10 mg/L FITC–gentamicin sulphate

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Mean ± s.d.</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unstained, CF(B)</td>
<td>2.13 ± 1.82</td>
<td>1.96</td>
</tr>
<tr>
<td>unstained, non-CF(W)</td>
<td>2.13 ± 1.82</td>
<td>1.96</td>
</tr>
<tr>
<td>FITC, CF(B)</td>
<td>8.41 ± 11.84</td>
<td>6.61</td>
</tr>
<tr>
<td>FITC, non-CF(W)</td>
<td>4.39 ± 9.29</td>
<td>3.19</td>
</tr>
</tbody>
</table>

| Treatment b |             |        |
| unstained, CF(A) | 2.11 ± 1.99 | 1.95   |
| unstained, non-CF(Z) | 2.11 ± 1.99 | 1.95   |
| FITC–gentamicin, CF(A) | 11.12 ± 14.46 | 8.66   |
| FITC–gentamicin, non-CF(Z) | 4.29 ± 5.35 | 2.74   |

relative concentrations of adenosine nucleotides were (μmol/g cell protein ± s.d.) (i) in CF cells: ATP, 7.1 ± 0.4; ADP, 7.1 ± 0.4; AMP, undetectable; (ii) in non-CF cells: ATP, 4.6 ± 0.3; ADP, 3.4 ± 0.2; AMP, 1.4 ± 0.2.
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References


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