

Activation of $\Delta F508$ CFTR in a cystic fibrosis respiratory epithelial cell line by 4-phenylbutyrate, genistein and CPX

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Activation of $\Delta F508$ -CFTR in respiratory epithelial cells by 4-phenylbutyrate, genistein and CPX. C. Andersson, G.M. Roomans. ©ERS Journals Ltd 2000.

ABSTRACT: The cellular basis of cystic fibrosis (CF) is a defect in a cyclic adenosine monophosphate (cAMP)-activated chloride channel (CF transmembrane conductance regulator) in epithelial cells that leads to decreased chloride ion transport and impaired water transport across the cell membrane. This study investigated whether it was possible to activate the defective chloride channel in cystic fibrosis respiratory epithelial cells with 4-phenylbutyrate (4PBA), genistein and 8-cyclopentyl-1,3-dipropylxanthine (CPX).

The CF bronchial epithelial cell line CFBE410-, which expresses the $\Delta F508$ mutation, was treated with these agents and loss of Cl^- , indicating Cl^- efflux, measured by X-ray microanalysis.

8-bromo-cAMP alone did not induce Cl^- efflux in CFBE410- cells, but after incubation with 4PBA a significant efflux of Cl^- occurred. Stimulation of cells with a combination of genistein and cAMP also induced Cl^- efflux, whereas a combination of pretreatment with 4PBA and a combined stimulation with genistein and cAMP induced an even larger Cl^- efflux. Cl^- efflux could also be stimulated by CPX, but this effect was not enhanced by 4PBA pretreatment.

The $\Delta F508$ mutation leads to impaired processing of the cystic fibrosis transmembrane conductance regulator. The increased efflux of chloride after 4-phenylbutyrate treatment can be explained by the fact that 4-phenylbutyrate allows the $\Delta F508$ cystic fibrosis transmembrane conductance regulator to escape degradation and to be transported to the cell surface. Genistein and 8-cyclopentyl-1,3-dipropylxanthine act by stimulating chloride ion efflux by increasing the probability of the cystic fibrosis transmembrane conductance regulator being open. The combination of 4-phenylbutyrate and genistein may be useful in a potential pharmacological therapy for cystic fibrosis patients with the $\Delta F508$ mutation.

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Cystic fibrosis (CF) is a monogenic disease caused by a mutation in the gene that codes for the CF transmembrane conductance regulator (CFTR). The CFTR functions as a cyclic adenosine monophosphate (cAMP)-activated chloride channel that is regulated by phosphorylation of the regulatory domain by protein kinase A and C and by binding and hydrolysis of adenosine triphosphate at the two nucleotide-binding domains. The defective protein causes impaired chloride ion transport in epithelial cells. $\Delta F508$ CFTR, the most common mutation in CF, results in a partially functional chloride channel with a decreased probability of being open. However, the mutation causes the retention of the CFTR in the endoplasmic reticulum (ER) so that it does not traffic properly to the plasma membrane.

There are several potential ways of bypassing this trafficking defect: stabilization of the protein by chemical chaperones [1], disruption of the interaction between $\Delta F508$ CFTR and a molecular chaperone, *e.g.* heat shock protein (Hsp)70 or calnexin that retains the mutant CFTR in the ER [2], overexpression of the mutant $\Delta F508$ CFTR to allow it to escape the ER control system [3], or transcriptional regulation of proteins involved in the ER control system.

4-phenylbutyrate (4PBA) allows the $\Delta F508$ CFTR to be directed to the plasma membrane, possibly *via* effects on Hsp70 levels, assuming that 4PBA has the same effect as

butyrate [4]. 4PBA has been reported to correct the cAMP-mediated Cl^- transport in primary cultures of nasal polyp epithelia from CF patients and in a CF cell line [5]. It also induces CFTR function in the nasal epithelia of $\Delta F508$ -homozygous patients, as measured by nasal potential difference [6]. 4PBA can correct the trafficking defect, but correction of the chloride channel properties may also be necessary in clinical therapy. Genistein, a tyrosine kinase inhibitor abundant in legumes, is known to activate both wild-type and $\Delta F508$ CFTR by increasing its probability of being open, probably by direct interaction with the CFTR [7–10]. 8-cyclopentyl-1,3-dipropylxanthine (CPX) is an adenosine A_1 -receptor antagonist that activates the $\Delta F508$ CFTR and has been shown to bind to the nucleotide binding domain 1 of the CFTR [11].

It was hypothesized that genistein or CPX could enhance the effect of 4PBA on Cl^- secretion from CF airway epithelial cells.

Material and methods

Materials and solutions

8-bromo-cAMP, genistein, CPX, fibronectin, collagen and bovine serum albumin (BSA) were from Sigma Chemical Co. (St Louis, MO, USA). 4PBA was from

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Triple Crown America (Perkasie, PA, USA). Standard Ringer's solution consisted of: 140 mM NaCl, 5 mM KCl, 5 mM (2-hydroxyethyl)-*N*-piperazine *N*-2 ethanesulphonic acid (HEPES), 1 mM MgCl₂, 1.5 mM CaCl₂ and 5 mM glucose, pH 7.4.

Cell culture

The CFBE41o- and 16HBE14o- cell lines, the generous gift of D.C. Gruenert, (University of California, San Francisco, CA, USA), were cultured in minimal essential medium with glutamax 524 mg·L⁻¹ (Gibco, BRL/Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum, 100 U·mL⁻¹ penicillin and 100 µg·mL⁻¹ streptomycin sulphate in 5% carbon dioxide in air at 37°C. The culture flasks were coated with a solution containing 0.01 mg·mL⁻¹ fibronectin, 0.029 mg·mL⁻¹ collagen and 0.1 mg·mL⁻¹ BSA for ≥1 h before seeding the cells. The medium was changed twice weekly.

X-ray microanalysis

Cells were cultured to confluence on titanium grids covered with a carbon-coated Formvar film (Merck, Darmstadt, Germany) coated with the fibronectin, collagen, and BSA solution (see *Cell culture*). Cells were incubated with or without 4PBA in Dulbecco's modified Eagle's medium:Ham's F12 medium (1:1) with glutamax (524 mg·L⁻¹), 2% Ultrosor G (Gibco, BRL/Life Technologies) and 50 µg·mL⁻¹ gentamicin for 72 h. The cells were then stimulated with 5 mM 8-bromo-cAMP or 5 mM 8-bromo-cAMP and 50 µM genistein for 3 min or with 30 nM CPX for 15 min in standard Ringer's solution. The cells were rinsed with cold distilled water for some seconds before being frozen in liquid propane cooled by liquid nitrogen (-180°C) and freeze-dried overnight under vacuum at -120°C. The freeze-dried cells were coated with a conductive carbon layer before analysis. Genistein and CPX were prepared from stock solutions in dimethyl sulphoxide (DMSO). The concentration of DMSO to which the cells were exposed was ≤1%. No changes in the concentrations of the various elements measured were seen in the cells after exposure to 1% DMSO for 3 and 15 min. Cells incubated with standard Ringer's solution (3 or 15 min) were used as control.

Analysis was performed using a Hitachi H7100 electron microscope (Hitachi, Tokyo, Japan) in the scanning transmission electron microscopy mode at 100 kV with an Oxford Instruments ISIS energy-dispersive spectrometer system (Oxford Instruments, Oxford, UK). Quantitative analysis was performed by comparing the ratio of the characteristic peak and the background under the peak (P:B) with the P:B of standards consisting of a gelatin/glycerol matrix with mineral salts in known concentrations [12]. Spectra were acquired for 100 s and each cell was analysed only once.

Statistical analysis

One-way analysis of variance and Newman-Keuls multiple comparison *post hoc* test were used to evaluate statistical significance when more than two groups were compared. When two groups were compared, an unpaired t-test was used. Significance was attributed to a p-value of <0.05.

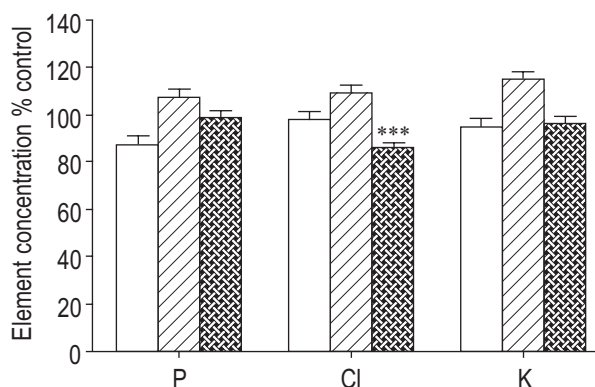


Fig. 1. – Effects of 8-bromo-cyclic adenosine monophosphate (8-bromo-cAMP), 4-phenylbutyrate (4PBA, ▩) and the combination of 8-bromo-cAMP and 4PBA (▨) on phosphorus, chlorine and potassium levels in CFBE41o- cells. The data are presented as mean±SEM from three to six experiments with 62 measurements on control cells, 28 on 8-bromo-cAMP-stimulated cells, 45 on 4PBA-treated cells and 77 on cells treated with the combination of 4PBA and 8-bromo-cAMP. ***: $p < 0.001$ versus control.

Results

Incubation with 4PBA or stimulation with 8-bromo-cAMP alone did not affect the cellular content of phosphorus, chlorine or potassium in CFBE41o- cells, but, after incubation with 4PBA, 8-bromo-cAMP caused a significant decrease in the chlorine content (fig. 1). An X-ray spectrum of the cells is shown in figure 2. Stimulation of cells with a combination of genistein and 8-bromo-cAMP reduced the chlorine content of the cells, and a combination of pretreatment with 4PBA and stimulation with genistein and cAMP together induced an even larger decrease in chlorine content (fig. 3). CPX significantly reduced the chlorine content but 4PBA did not enhance this effect (fig. 4). The chlorine content of the control cells was stable for ≥15 min. The data for phosphorous, an element for which no significant changes were expected, are provided as an "internal control".

In order to determine whether the secretion of Cl⁻ in CF cells is of physiological relevance, a normal human bronchial epithelial cell line expressing the CFTR, 16HBE14o-, was stimulated with 8-bromo-cAMP (fig. 5). The decrease

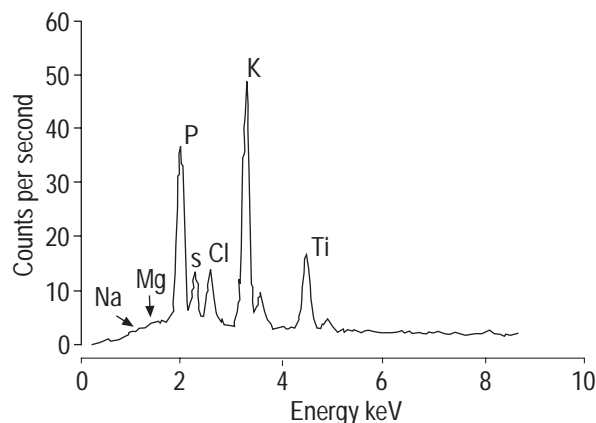


Fig. 2. – X-ray spectrum of CFBE41o- cells showing the characteristic peaks for sodium, magnesium, phosphorus, sulphur, chlorine, potassium and titanium. The Ti peak is due to the grid.

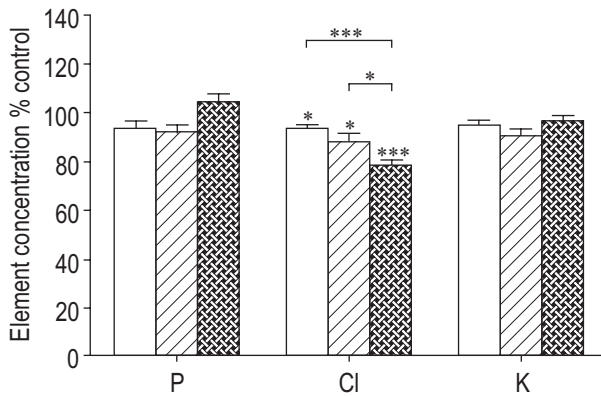


Fig. 3. – Effects of 4-phenylbutyrate (4PBA), genistein and 8-bromo-cyclic adenosine monophosphate (cAMP) (8-bromo-cAMP) on phosphorus, chlorine and potassium levels in CFBE41o- cells. Data are presented as mean \pm SEM from four to eight experiments with 96 measurements on 8-bromo-cAMP and genistein (\square), 59 on cells stimulated with 4PBA and cAMP (▨) and 87 on cells stimulated with 4PBA, 8-bromo-cAMP and genistein (▩). *: $p < 0.05$; ***: $p < 0.001$ versus 8-bromo-cAMP or between columns indicated.

in Cl^- after stimulation with 8-bromo-cAMP in 16HBE-140- cells was 26%. This can be compared with a 15% decrease after incubation with 4PBA, a 22% decrease on stimulation with genistein and cAMP after incubation with 4PBA, and a 13% decrease after stimulation with CPX (fig. 6).

Discussion

The experimental results in this study indicate that correction of the cAMP-dependent Cl^- secretion by treatment with 4PBA can be further improved by genistein, an agent that increases the probability of the $\Delta F508$ CFTR being open. Also, CPX increased Cl^- secretion in CFBE41o- cells.

It was found that the transcriptional regulator 4PBA increased Cl^- secretion in the CFBE41o- cell line, which expresses the $\Delta F508$ CFTR mutation. This is consistent with previous results with another CF cell line and primary

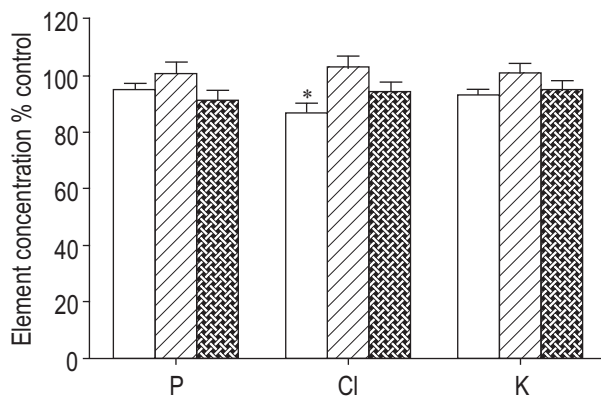


Fig. 4. – Effects of 8-cyclopentyl-1,3-dipropylxanthine (CPX) and 4-phenylbutyrate (4PBA) on CFBE41o- cells. Data are presented as mean \pm SEM from four to six experiments with 43 measurements on control cells, 60 on cells stimulated with CPX (\square), 43 on cells treated with 4PBA and 38 on cells treated with 4PBA and CPX (▩). *: $p < 0.05$ versus control.

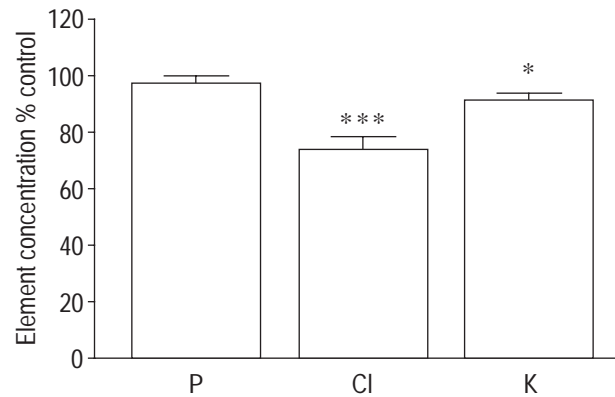


Fig. 5. – Effects of 8-bromo-cyclic adenosine monophosphate (8-bromo-cAMP) on 16HBE140- cells. Data are presented as mean \pm SEM from three experiments with 26 measurements on control cells and 48 on 8-bromo-cAMP-stimulated cells. *: $p < 0.05$; ***: $p < 0.001$ versus control.

CF epithelial cell cultures [5]. The effect of the xanthine CPX and the isoflavone genistein on the CF cell line was investigated and this treatment combined with 4PBA to see whether the effect of 4PBA could be further improved.

CPX induced Cl^- secretion in the CFBE41o- cell line, which is consistent with previous studies on CF cell lines [13], but an increase of this effect after incubation with 4PBA was not seen in the present study. The mechanism by which CPX activates Cl^- efflux is not clear. CPX has been shown to bind to the first nucleotide-binding fold in $\Delta F508$ CFTR [11]. Other studies argue against a direct activation of CFTR [14] and suggest that the CPX-induced Cl^- efflux is related to CPX induced changes in intracellular pH [14, 15]. Since 4PBA did not increase the effect of CPX, the present data do not support a direct effect of CPX on $\Delta F508$ CFTR.

Genistein potentiates cAMP-dependent activation of $\Delta F508$ CFTR [8]. The present results confirm that genistein induces the cAMP-dependent activation of $\Delta F508$

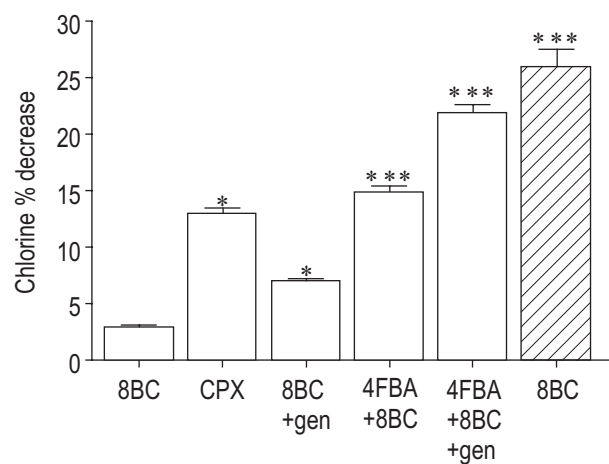


Fig. 6. – Effect of stimulation with various drugs on chlorine concentration in cystic fibrosis (\square) and normal (▩) cells. Data are presented as mean \pm SEM. 8BC: 8-bromo-cyclic adenosine monophosphate; CPX: 8-cyclopentyl-1,3-dipropylxanthine; gen: genistein; 4PBA: 4-phenylbutyrate. *: $p < 0.05$; ***: $p < 0.001$ versus control.

CFTR and suggests further that it is able to increase the Cl⁻ secretion achieved after incubation with 4PBA.

It has previously been shown, by X-ray microanalysis, that loss of cellular chlorine (equivalent to Cl⁻ efflux) occurs after stimulation with cAMP (analogues) in, for example, airway epithelial cells [16], tracheal gland cells [17] and sweat gland cells [18] containing the CFTR channel. This loss was absent in airway epithelial cells from CF patients [16], tracheal gland cells treated with antisense CFTR [17] and sweat gland cells and tracheal gland cells in the presence of chloride channel blockers [17–19]. Therefore, X-ray microanalysis is a useful technique for determining Cl⁻ efflux. The technique gives data in mmol·kg dry weight⁻¹, which may make the data difficult to interpret in a physiological context. However, by making assumptions about the water content, the data can be converted by approximation to millimolarity, which is the more usual unit for physiological investigations.

The chlorine and potassium content of the CFBE410-cell line was ~190 mmol·kg dry weight⁻¹ and ~900 mmol·kg dry weight⁻¹ respectively. Assuming that ~80% of the cell is water, these values are equivalent to ~48 mM intracellular Cl⁻ and 225 mM intracellular K⁺. The 26% decrease in Cl⁻ in the normal cells expressing the CFTR after 8-bromo-cAMP stimulation is therefore equivalent to a 12 mM decrease in Cl⁻. It is expected that Cl⁻ efflux is accompanied by K⁺ efflux. In all experiments, the potassium concentrations in the cells that show chloride efflux are lower than in the control cells by ~40–50 mmol·kg dry weight⁻¹, or practically the same as the Cl⁻ efflux. This means that there is no need to invoke mechanism other than Cl⁻ efflux accompanied by a stoichiometric K⁺ efflux to maintain electroneutrality and it is not necessary to assume, for example, Cl⁻/bicarbonate exchange. However, this efflux of K⁺ is so small relative to the high K⁺ content of the cells (~5%) that a significant decrease in potassium content could only be seen in the experiment shown in figure 5.

The effect achieved by 4PBA, genistein and CPX on the CF cell line was compared with the effect of 8-bromo-cAMP on human bronchial epithelial cells expressing the wild-type CFTR. With 4PBA (15% decrease), genistein (7% decrease) and CPX (13% decrease) the reduction in Cl⁻ concentration in the cells is less than the 26% decrease achieved by cAMP in the normal cells. However, combined treatment with 4PBA and genistein, with a reduction in Cl⁻ concentration of 22%, is comparable with the decrease achieved in normal cells (fig. 6). Many groups have shown that genistein increases the probability of the ΔF508 CFTR being open and have suggested a direct action of genistein on the CFTR [7–9]. Genistein increases the phosphorylation level of the regulatory domain [8, 20] without increasing cAMP levels or protein kinase A activity [20, 21]. Furthermore, it prevents deactivation of the channel after removal of cAMP-inducing stimulation [21, 22]. This can be explained by inhibition of the dephosphorylation of the regulatory domain that inactivates the channel. Genistein increases the phosphorylation level of the CFTR but also increases the activity of the channel independent of the phosphorylation level [8].

4-phenylbutyrate directs more ΔF508 cystic fibrosis transmembrane conductance regulator to the cell membrane. Genistein can act on these channels to increase the

open time and supplement the effect achieved with 4-phenylbutyrate. 4-phenylbutyrate is already in clinical use as an ammonia scavenger to treat urea cycle enzyme deficiencies. Genistein is a soy isoflavone that has been suggested to prevent cancer [23, 24] and atherosclerosis [25]. It would therefore be interesting to consider the therapeutic potential of a combination of 4-phenylbutyrate and genistein or genistein-like drugs as a pharmacological treatment for cystic fibrosis.

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