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RESEARCH PAPER

Prolonged treatment of cells with genistein modulates the expression and function of the cystic fibrosis transmembrane conductance regulator

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Background and purpose: Cystic fibrosis (CF) is caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. In the search for new CF therapies, small molecules have been identified that rescue the defective channel gating of CF mutants (termed CFTR potentiators). Here, we investigate the long-term effects of genistein, the best-studied CFTR potentiator, on the expression and function of CFTR.
Experimental approach: We pre-treated baby hamster kidney (BHK) cells expressing wild-type or F508del-CFTR (the most common CF mutant) with concentrations of genistein that potentiate (30 μM) or inhibit (100 μM) CFTR function for 2 or 24 h at 37 °C before examining CFTR maturation, expression and single-channel activity.
Key results: Using the iodide efflux technique, we found that genistein pre-treatment failed to restore function to F508del-CFTR, but altered that of wild-type CFTR. Pre-treatment of cells with genistein for 2 h had little effect on CFTR processing, whereas pre-treatment for 24 h either augmented (30 μM genistein) or impaired (100 μM genistein) CFTR maturation. Using immunocytochemistry, we found that all genistein pre-treatments increased the localization of CFTR protein to the cell surface. However, following the incubation of cells with genistein (100 μM) for 2 h, individual CFTR Cl^- channels exhibited characteristics of channel block upon channel activation.
Conclusions and implications: Genistein pre-treatment alters the maturation, cell surface expression and single-channel function of CFTR in ways distinct from its acute effects. Thus, CFTR potentiators have the potential to influence CFTR by mechanisms distinct from their effects on channel gating.
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Abbreviations: BHK cells, baby hamster kidney cells; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; f_c, corner frequency; *i*, single-channel current amplitude; MBD, mean burst duration; NBD, nucleotide-binding domain; PKA, protein kinase A

Introduction

Cystic fibrosis (CF) is a life-shortening genetic disease common in Caucasian populations (Welsh *et al.*, 2001; Rowe *et al.*, 2005). The major cause of morbidity and mortality in CF is respiratory airway disease. Airway disease in CF has two components: first, defective epithelial ion transport prevents normal mucocilary clearance with the result that thick, sticky mucus, which favours bacterial binding, accumulates and obstructs the airways. Second, there is a localized failure of the host defence system in the respiratory airways leading to persistent bacterial infections (Welsh *et al.*, 2001; Rowe *et al.*, 2005). CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR; Riordan *et al.*, 1989), a Cl^- channel with complex regulation that plays an essential role in fluid and electrolyte transport across epithelia (Sheppard and Welsh, 1999; Welsh *et al.*, 2001). CFTR is assembled from five domains: two membrane-spanning domains that form an anion-selective pore, two nucleotide-binding domains (NBDs) that control channel gating and a unique regulatory domain, phosphorylation of which is a prerequisite for channel activity (Sheppard and Welsh, 1999; Gadsby *et al.*, 2006).

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To date, almost 1500 disease-causing mutations have been identified in the CFTR gene (see The Cystic Fibrosis Mutation Database; http://www.genet.sickkids.on.ca/cftr/). Most mutations are very rare. The exception is F508del, the deletion of a phenylalanine residue at position 508 of the CFTR sequence. This mutation accounts for about 70% of CF mutations worldwide and is associated with a severe disease phenotype (Welsh *et al*., 2001). The molecular basis for the F508del defect is protein misfolding, which causes the retention of the mutant protein by the endoplasmic reticulum (ER) quality control and rapid targeting for degradation by the ubiquitin–proteasome pathway (for review, see Amaral, 2005). Hence, the vast majority of F508del-CFTR is neither processed through the Golgi apparatus, where wild-type CFTR is glycosylated to form the mature protein, nor delivered to the plasma membrane. In native tissues, a small amount of F508del-CFTR reaches the cell membrane where it forms Cl^- channels with conduction and permeation properties in common with those of wild-type CFTR (for example, Dalemans *et al*., 1991; Denning *et al*., 1992). However, the pattern of channel gating of the F508del-CFTR Cl^- channel, which is characterized by infrequent bursts of channel openings separated by long closures of prolonged duration, differs strikingly from that of wild-type CFTR (Dalemans *et al*., 1991; Sheppard and Welsh, 1999).

Knowledge of how F508del-CFTR disrupts CFTR-mediated Cl^- transport in CF is leading to rational new approaches to therapy (Amaral and Kunzelmann, 2007; Cai *et al*., 2007). Restoration of channel function to F508del-CFTR requires the use of both a drug to deliver the mutant protein to its correct cellular location (termed a CFTR corrector) and a drug to rescue defective channel gating (termed a CFTR potentiator). To date, most studies of CFTR potentiators have examined the acute effects of these agents on CFTR Cl^- channel function. However, when used as drugs, CFTR-expressing cells will be exposed to CFTR potentiators for prolonged periods.

To explore the long-term effects of CFTR potentiators on the CFTR Cl^- channel, the aim of the present study was to investigate the effects of prolonged exposure to genistein on the expression, localization and function of CFTR. We selected for study this flavonoid because, as the most-studied CFTR potentiator, its mechanism of action is best understood (Hwang and Sheppard, 1999; Lansdell *et al*., 2000; Ai *et al*., 2004). For practical reasons, we employed baby hamster kidney (BHK) cells engineered to express high levels of CFTR protein rather than polarized epithelial cells expressing endogenous CFTR. Using the iodide efflux technique, we investigated the effects of prolonged genistein exposure on the activity of large populations of wild-type and F508del-CFTR in intact BHK cells. Then using biochemical and cell biological techniques, we explored the long-term effects of genistein on the maturation and expression of CFTR protein and with electrophysiological methods, we examined the drug's long-term effects on the single-channel function of CFTR. We discovered that genistein pretreatment altered the processing, cell surface localization and single-channel function of wild-type CFTR in ways distinct from its acute effects on the channel. Thus, CFTR potentiators have the potential to influence CFTR by mechanisms distinct from their effects on channel gating.

Materials and methods

Cells and cell culture

For this study, we used untransfected BHK cells and BHK cells stably expressing either wild-type or F508del human CFTR (Farinha *et al*., 2002). Transfected BHK cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's Medium and Ham's F-12 nutrient medium supplemented with 5% $v\,v^{-1}$ fetal calf serum, $100\,U\,ml^{-1}$ penicillin and $100\,mg\,ml^{-1}$ streptomycin (all from Invitrogen Ltd., Paisley, UK) and $200\,\mu g\,ml^{-1}$ methotrexate (AAH Pharmaceuticals Ltd., Coventry, UK) at 37 °C in a humidified atmosphere of 5% CO_2. Untransfected BHK cells were cultured using the same media, but without added methotrexate. In some experiments, BHK cells expressing F508del-CFTR were cultured at 28 °C to overcome the processing defect of this mutation and promote its delivery to the cell membrane (Denning *et al*., 1992).

For iodide efflux, pulse-chase and immunoprecipitation experiments, cells were seeded onto 60 mm plastic culture dishes and used when dishes were confluent (functional studies, 3–4 days (37 °C incubations) or 4–5 days (28 °C incubations); biochemical studies, 2 days). For immunocytochemistry, cells were seeded onto eight-well chamber slides (Nalge Nunc, Roskilde, Denmark) and grown for 1 day at 37 °C prior to use. For experiments using excised inside-out membrane patches, cells were seeded onto glass coverslips and used within 60 h. To investigate the long-term effects of genistein, we pre-incubated cells for either 2 or 24 h at 37 °C in media containing either a specific concentration of genistein (30 or 100 μM; Sigma-Aldrich, Gillingham, UK) or an equivalent volume of the vehicle dimethyl sulphoxide (DMSO). As a control, we used the tyrosine kinase inhibitor tyrphostin 47 (100 μM; Sigma-Aldrich), testing its action under identical conditions to those used for genistein (100 μM).

Iodide efflux experiments

We measured CFTR-mediated iodide efflux, as described by Lansdell *et al*. (1998), using the cAMP agonist forskolin (10 μM) and the CFTR potentiator genistein (50 μM; Sigma-Aldrich). We used genistein (50 μM) to potentiate CFTR activity in iodide efflux experiments for two reasons: (i) the phosphorylation dependence of genistein's action on CFTR (see Lansdell *et al*., 2000) and (ii) the restricted access of extracellular genistein to its binding site on the NBDs (see Discussion). Prior to commencing experiments, BHK cells were incubated for 1 h in loading buffer containing (in mM): 136 NaI, 3 KNO_3, 2 $Ca(NO_3)_2$, 20 HEPES and 11 glucose, pH 7.4 with 1 M NaOH; osmolarity, 299 ± 0.48 mosM ($n = 36$) and then washed thoroughly with efflux buffer (136 mM $NaNO_3$ replacing 136 mM NaI in the loading buffer; osmolarity, 291 ± 0.24 mosM ($n = 60$)). The amount of iodide in each sample of efflux buffer was determined using an iodide-selective electrode (Russell pH Ltd., Auchtermuchty, UK).

BHK cells were loaded and experiments were performed at room temperature (~23 °C).

Pulse-chase and immunoprecipitation experiments

After genistein pretreatment, cells were starved for 30 min in methionine-free α-modified Eagle's medium (Invitrogen Ltd.) before being radiolabelled for 25 min in the same medium supplemented with 150 μCi ml^{-1} [^{35}S]methionine (ICN Biomedicals, Irvine, CA, USA) (Farinha *et al*., 2002). For the chase (0, 0.5, 1, 2 and 3 h), the labelling medium was replaced by α-modified Eagle's medium supplemented with fetal bovine serum (8% v v^{-1}; Invitrogen Ltd.) and non-radioactive methionine (1 mM; Sigma-Aldrich). Cells were then lysed in radioimmunoprecipitation assay buffer (1 ml) containing deoxycholic acid (1% w v^{-1}; Sigma-Aldrich), Triton X-100 (1% v v^{-1}; GE Healthcare Bio-Sciences, Uppsala, Sweden), SDS (0.1% w v^{-1}; Invitrogen Ltd.), Tris (50 mM; pH 7.4; Sigma-Aldrich) and NaCl (150 mM). CFTR protein was immunoprecipitated as described by Jensen *et al*. (1995) after centrifugation of samples at 14 000 *g* for 30 min at 4 °C. To detect specifically CFTR, the supernatant was incubated overnight with 1.5 μg of the anti-CFTR monoclonal antibody M3A7, which recognizes NBD2 and the C terminus of CFTR (residues 1197–1480; Chemicon, Temecula, CA, USA) at 4 °C and then protein–G agarose beads (25 μg; Roche, Basel, Switzerland) were added for a further 4 h at 4 °C. Beads were washed four times using radioimmunoprecipitation assay buffer (1 ml) and protein eluted for 1 h at room temperature with cracking buffer (80 μl) containing dithiothreitol (0.5 mM; Sigma-Aldrich), bromophenol blue (0.001% w v^{-1}), glycerol (5% v v^{-1}), SDS (1.5% w v^{-1}) and Tris (31.25 mM), pH 6.8. Samples were separated electrophoretically on 7% w v^{-1} polyacrylamide gels. Then, gels were pre-fixed (methanol (30% v v^{-1}) and acetic acid (10% v v^{-1})) for 30 min, washed thoroughly in water and soaked in salicylic acid (1 M) for 1 h. After drying at 80 °C under vacuum for 2 h, gels were exposed to X-ray film (Fujifilm Medical Systems, Stamford, CT, USA). Fluorograms of gels were digitized (Sharp JX-330; Sharp Europe, Hamburg, Germany) and integrated peak areas were determined using ImageMaster software (GE Healthcare Bio-Sciences).

Immunocytochemistry

Immunocytochemistry was performed as described previously (Mendes *et al*., 2005). In brief, following drug treatment, BHK cells were rinsed twice with cold phosphate-buffered saline (PBS) and fixed with ice-cold methanol for 10 min. After two washes with PBS at room temperature, BHK cells were permeabilized with Triton X-100 (0.25% w v^{-1}) in PBS for 20 min and washed three times in PBS (5 min each at room temperature). Nonspecific staining was prevented by blocking with BSA (1% w v^{-1}) in PBS for 30 min prior to overnight incubation at 4 °C with a 1:25 dilution of the anti-CFTR monoclonal antibody 24-1, which recognizes the C terminus of CFTR (residues 1377–1480; R&D Systems, Minneapolis, MN, USA). Cells were then washed three times with PBS (10 min each at room temperature) and incubated with a 1:60 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Chemicon) for 45 min at room temperature. Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) to stain nuclei and covered with a glass coverslip. Immunofluorescence staining was observed and recorded on an Axioskop fluorescence microscope (Zeiss, Jena, Germany) with Power Gene 810/Probe & CGH software system (Perceptive Scientific Instruments, Chester, UK).

Electrophysiology

CFTR Cl^- currents were recorded in excised inside-out membrane patches using an Axopatch 200A patch-clamp amplifier (Molecular Devices Corp., Union City, CA, USA) and pCLAMP data acquisition and analysis software (versions 6.0.4 and 8.1.0; Molecular Devices Corp.) as described previously (Sheppard and Robinson, 1997; Lansdell *et al*., 2000). The established sign convention was used throughout; currents produced by positive charge moving from intra- to extracellular solutions (anions moving in the opposite direction) are shown as positive currents.

The pipette (extracellular) solution contained (mM): 140 *N*-methyl-D-glucamine, 140 aspartic acid, 5 $CaCl_2$, 2 $MgSO_4$ and 10 *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES), pH 7.3 with Tris ([Cl^-], 10 mM; osmolarity, 281 ± 0.5 mosM ($n = 4$). The bath (intracellular) solution contained (mM): 140 *N*-methyl-D-glucamine, 3 $MgCl_2$, 1 CsEGTA and 10 *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid, pH 7.3 with HCl, ([Cl^-], 147 mM; $[Ca^{2+}]_{free}$, $<10^{-8}$ M; osmolarity, 279 ± 0.5 mosM ($n = 7$) and was maintained at 37 °C.

After their excision, membrane patches were voltage-clamped at −50 mV and CFTR Cl^- channels were activated by the addition of ATP (1 mM; Sigma-Aldrich) and the catalytic subunit of protein kinase A (PKA; 75 nM; Promega UK, Southampton, UK) to the intracellular solution within 5 min of patch excision. In this study, we used membrane patches containing large numbers of active channels from cells pretreated with genistein. We examined the effects of genistein pretreatment on the single-channel behaviour of CFTR by studying individual CFTR Cl^- channels active in the presence of ATP (1 mM) prior to phosphorylation by PKA.

CFTR Cl^- currents were initially recorded on digital audiotape using a digital tape recorder (Biologic Scientific Instruments, model DTR-1204; Intracel Ltd., Royston, UK) at a bandwidth of 10 kHz. On playback, records were filtered with an eight-pole Bessel filter (model 902LPF2; Frequency Devices Inc., Haverhill, MA, USA) at a corner frequency of 500 Hz and acquired using a Digidata 1200 interface (Molecular Devices Corp.) and pCLAMP software at a sampling rate of 5 kHz. For the purpose of illustration, single-channel records were filtered at 500 Hz and digitized at 1 kHz.

We measured single-channel current amplitude (*i*) using cursor measurements. We calculated mean burst duration (MBD) and frequency of channel opening using membrane patches that contained only bursts of single-level openings with no superimposed openings that were separated from one another by a minimum of 20 ms in the presence of ATP

(1 mM) as described previously (Carson *et al*., 1995; Cai *et al*., 2006).

Noise analysis

To study genistein-induced changes in gating kinetics, we applied the spectral analysis technique to macroscopic CFTR Cl^- currents using pCLAMP software (version 8.1.0; Lindemann and Van Driessche, 1977; Venglarik *et al*., 1996). We filtered records with an eight-pole Bessel filter (Frequency Devices Inc.; model 902LPF2) at a corner frequency of 5 kHz and acquired data using a Digidata 1200 interface (Molecular Devices Corp.) and pCLAMP software at a sampling rate of 20 kHz. Digitized records were partitioned into short segments of 8192 points (409.6 ms) and multiplied point-by-point using a Von Hanning window function before Fourier transformation. Power density spectra were calculated and averaged using a minimum of 100 segments of data. We determined the power density spectrum of background noise when CFTR Cl^- currents were quiescent and subtracted this from those of CFTR Cl^- currents. We fitted power density spectra with the sum of either two or three Lorentzian functions using the Levenberg–Marquardt least-squares method. The Lorentzian functions used had the form:

$$S(f) = \sum_{i=1}^{n} S_{(0)i}/[1 + (f/f_c)^2]$$

where i indicates the Lorentzian component, $S(f)$ is the power density spectrum at the frequency of f, $S_{(0)i}$ is the zero-frequency asymptote of Lorentzian component i and f_c is the corner frequency of the component i. We chose a frequency range from 10 Hz to 3 kHz for the fitting of Lorentzian functions because tolbutamide block of CFTR introduced a high-frequency Lorentzian component into power density spectra ($f_c = 650$ Hz; Venglarik *et al*., 1996).

Statistics

Results are expressed as means ± s.e.mean of n observations. To test for differences between groups of data, an ANOVA was used. To compare only two sets of data, we used Student's t-test. Differences were considered statistically significant when $P < 0.05$. Tests were performed using either Microsoft Excel or SigmaStat (version 2.03; Jandel Scientific GmbH, Erkrath, Germany).

Reagents

Forskolin was dissolved in methanol, whereas genistein and tyrphostin 47 were dissolved in DMSO. Stock solutions of forskolin, genistein and tyrphostin 47 were stored at −20 °C, while that for ATP was prepared immediately before each experiment and stored at +4 °C. Stock solutions were diluted in either BHK media or intracellular solution to achieve final concentrations immediately before use. Precautions against light-sensitive reactions were observed when using genistein and tyrphostin 47. Acute treatment with DMSO did not affect the Cl^- channel activity of CFTR (Sheppard and Robinson, 1997).

Results

Pretreating cells with genistein fails to rescue the function of F508del-CFTR, but alters that of wild-type CFTR

To investigate the long-term effects of CFTR potentiators on the CFTR Cl^- channel, we selected for the study the flavonoid genistein that potentiates robustly CFTR channel gating (Hwang *et al*., 1997), and treated cells with the drug for 2 or 24 h prior to evaluating CFTR expression and function. Because elevated concentrations of genistein inhibit CFTR, we tested concentrations of genistein that both potentiate (30 μM) and inhibit (100 μM) the CFTR Cl^- channel (Wang *et al*., 1998; Lansdell *et al*., 2000). To explore whether genistein pretreatment alters the cell surface expression of CFTR, we used BHK cells engineered to express high levels of wild-type and F508del-CFTR hereafter termed BHK-wt-CFTR and BHK-F508del-CFTR cells, respectively. As a control, we studied untransfected BHK cells.

We began by assessing the acute effects of genistein on the function of a large population of CFTR Cl^- channels in intact cells using the iodide efflux technique. At 37 °C, the cAMP agonist forskolin (10 μM) and genistein (50 μM) stimulated a large transient efflux of iodide from BHK-wt-CFTR cells, but did not have any effect on either BHK-F508del-CFTR or untransfected BHK cells (Figure 1a). Genistein (50 μM) enhanced the magnitude of CFTR-mediated iodide efflux stimulated by forskolin (10 μM) sixfold and accelerated the time to peak 2.5-fold (Figure 1b inset). At 28 °C, BHK-F508del-CFTR cells generated a small sustained CFTR-mediated iodide efflux, consistent with the delivery of some F508del-CFTR protein to the cell membrane at this temperature (Denning *et al*., 1992), while the magnitude of CFTR-mediated iodide efflux from BHK-wt-CFTR cells was attenuated slightly, but not significantly ($P = 0.07$; Figure 1a). As a further control, we pretreated untransfected BHK cells, BHK-wt-CFTR and BHK-F508del-CFTR cells with DMSO (0.1% v v^{-1}, the vehicle for genistein (100 μM)) for 24 h at 37 °C and demonstrated that it did not have any effect on the magnitude and time course of CFTR-mediated iodide efflux ($P > 0.05$; Figure 1b).

Next, we analysed the effects of genistein pretreatment on CFTR function using the same approach. Figures 1c and d summarize the effects of incubating BHK-wt-CFTR and BHK-F508del-CFTR cells with genistein (30 μM) at 37 °C for either 2 or 24 h, respectively. These pretreatments failed to rescue CFTR function in BHK-F508del-CFTR cells, but altered CFTR-mediated iodide efflux in BHK-wt-CFTR cells (Figures 1c and d). Figure 1c shows that pretreatment with genistein (30 μM) for 2 h attenuated the magnitude of iodide efflux, albeit not significantly ($P = 0.39$) and accelerated the response ($P = 0.03$). By contrast, pre-treatment with genistein (30 μM) for 24 h accentuated the magnitude of iodide efflux, albeit not significantly ($P = 0.10$) and accelerated the response ($P < 0.01$; Figure 1d).

Figures 1e and f summarize the effects of incubating BHK-wt-CFTR and BHK-F508del-CFTR cells with genistein (100 μM) at 37 °C for either 2 or 24 h, respectively. These pretreatments did not have any effect on iodide efflux from either BHK-F508del-CFTR cells or untransfected BHK cells (Figures 1e and f). However, they attenuated markedly CFTR-mediated iodide

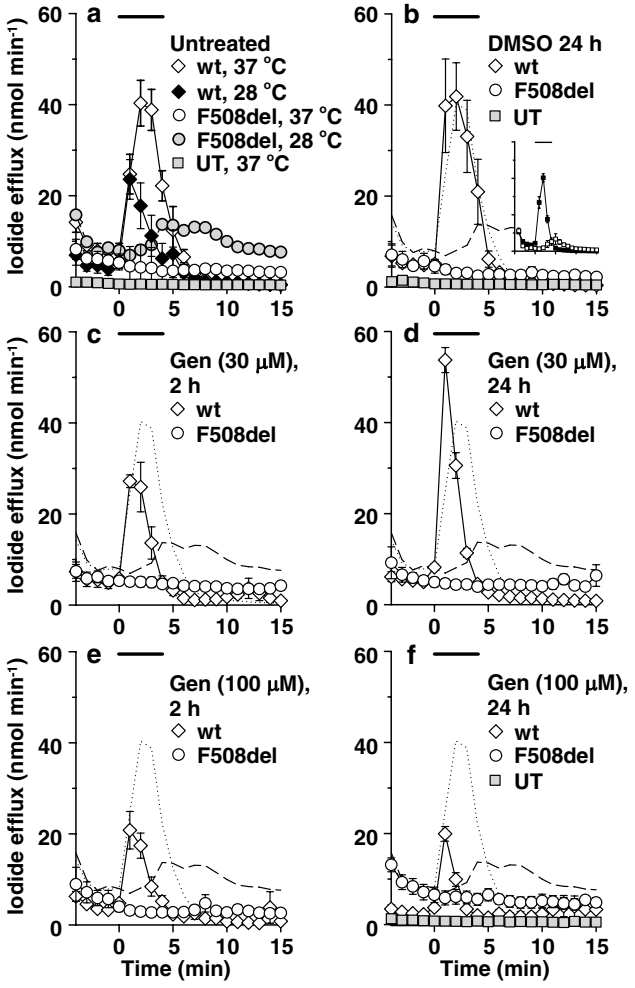

Figure 1 The effects of genistein incubation on iodide efflux from baby hamster kidney (BHK) cells expressing wild-type and F508del-CFTR. Data show the time course of iodide efflux from BHK-wt-CFTR and BHK-F508del-CFTR cells pretreated with genistein (30 or 100 μM) for 2 or 24 h. Diamonds and circles represent data from BHK-wt-CFTR and BHK-F508del-CFTR cells, respectively, while grey squares denote data from untransfected BHK cells (UT). (**a**, **b**) Controls; (**a**) cells cultured at 37 and 28 °C. (**b**) Cells pretreated for 24 h with dimethyl sulphoxide (DMSO) (0.1% v v^{-1}), the vehicle for genistein (100 μM) at 37 °C. The inset compares the time course of iodide efflux from untreated BHK-wt-CFTR cells stimulated by forskolin (10 μM) alone (open squares) and forskolin (10 μM) plus genistein (50 μM) (filled squares). Abscissa: time, −5 to 15 min; ordinate: iodide efflux; 0–60 nmol min^{-1}. Values are means ± s.e.mean ($n=6$). (**c**, **d**) Cells pretreated with genistein (30 μM) for either 2 h (**c**) or 24 h (**d**) at 37°C. (**e**, **f**) Cells pretreated with genistein (100 μM) for either 2 h (**e**) or 24 h (**f**) at 37 °C. In (**b**–**f**), dotted and dashed lines indicate iodide efflux from untreated BHK-wt-CFTR cells grown at 37 °C and untreated BHK-F508del-CFTR cells grown at 28 °C, respectively, as shown in (**a**). During the periods indicated by bars, forskolin (10 μM) and genistein (50 μM) were added to the efflux buffer. Symbols and error bars are means ± s.e.mean ($n=6$) for each condition. Where not shown, error bars are smaller than symbol size.

efflux from BHK-wt-CFTR cells ($P<0.05$), while accelerating the response ($P<0.05$; Figures 1e and f). Thus, pretreating cells with concentrations of genistein that either potentiate or inhibit the CFTR Cl^- channel fails to rescue the activity of F508del-CFTR, but alters that of wild-type CFTR.

Genistein pretreatment modulates the processing efficiency of CFTR protein

In principle, genistein pretreatment might modulate CFTR activity in one or a combination of three ways: first, by altering the number of active channels at the cell surface (trafficking); second, by varying the current flow through open channels and third, by regulating channel gating and hence, open probability (P_o). To investigate how genistein pretreatment affects the number of CFTR Cl^- channels at the cell surface and their behaviour, we employed biochemical, cell biological and electrophysiological techniques.

When immunoprecipitation is used to investigate the biosynthesis of CFTR, two different forms of wild-type CFTR protein are visualized: an immature core-glycosylated form that is found in the ER (150 kDa; band B) and a mature fully glycosylated form that has been processed through the Golgi apparatus and delivered to the cell membrane (170–180 kDa; band C; Cheng *et al*., 1990). To evaluate the effects of genistein pretreatment on the turnover rate of band B and the efficiency of its processing into band C, we used pulse-chase analyses followed by CFTR immunoprecipitation.

Figure 2a demonstrates that both the immature and mature forms of CFTR were detected in untreated BHK-wt-CFTR cells as well as in those pretreated with either genistein (30 μM) or its vehicle DMSO (0.03% v v^{-1}) for 2 or 24 h at 37 °C. In contrast, only the immature form of CFTR was detected in untreated BHK-F508del-CFTR cells as well as those pretreated with either genistein (30 μM) or DMSO (0.03% v v^{-1}) for 24 h at 37 °C (Figure 2f). Pretreatment of BHK-wt-CFTR cells with either genistein (30 μM) or DMSO for 2 and 24 h had no effect on the turnover rate of band B (Figures 2b and c). Similarly, the turnover rate of band B was unaffected when BHK-F508del-CFTR cells were pretreated with these agents for 24 h (Figure 2g). However, these pretreatments altered the efficiency of processing of wild-type CFTR into band C. Figure 2d shows that at early chase times (30 and 60 min) the processing efficiency of wild-type CFTR was accelerated by 2 h pretreatments, albeit not significantly ($P>0.05$). Pretreatment for 24 h with DMSO did not have any effect, whereas genistein (30 μM) pretreatment enhanced wild-type CFTR processing efficiency, but not significantly ($P>0.05$; Figure 2e). We interpret these data to suggest that prolonged incubations with genistein (30 μM) appear to increase the maturation efficiency of wild-type CFTR.

Figure 3a demonstrates the presence of the immature and mature forms of CFTR in BHK-wt-CFTR cells pretreated with genistein (100 μM) for 2 h at 37 °C and the equivalent volume of DMSO for 2 or 24 h at 37 °C. In contrast, only the immature form of CFTR was detected in untreated BHK-F508del-CFTR cells as well as those pretreated with either genistein (100 μM) or DMSO (0.1% v v^{-1}) for 24 h at 37 °C (Figure 3f). Interestingly, after pretreatment with genistein (100 μM) for 24 h at 37 °C, CFTR appeared as a diffuse band of higher mobility than band C, but lower than that of band B (Figure 3a). Like the effects of low concentrations of the drug (Figures 2b and c), incubating BHK-wt-CFTR cells with either genistein (100 μM) or DMSO for 2 and 24 h did not have any effect on the turnover rate of band B (Figures 3b and c). The turnover rate of band B was also unaffected when

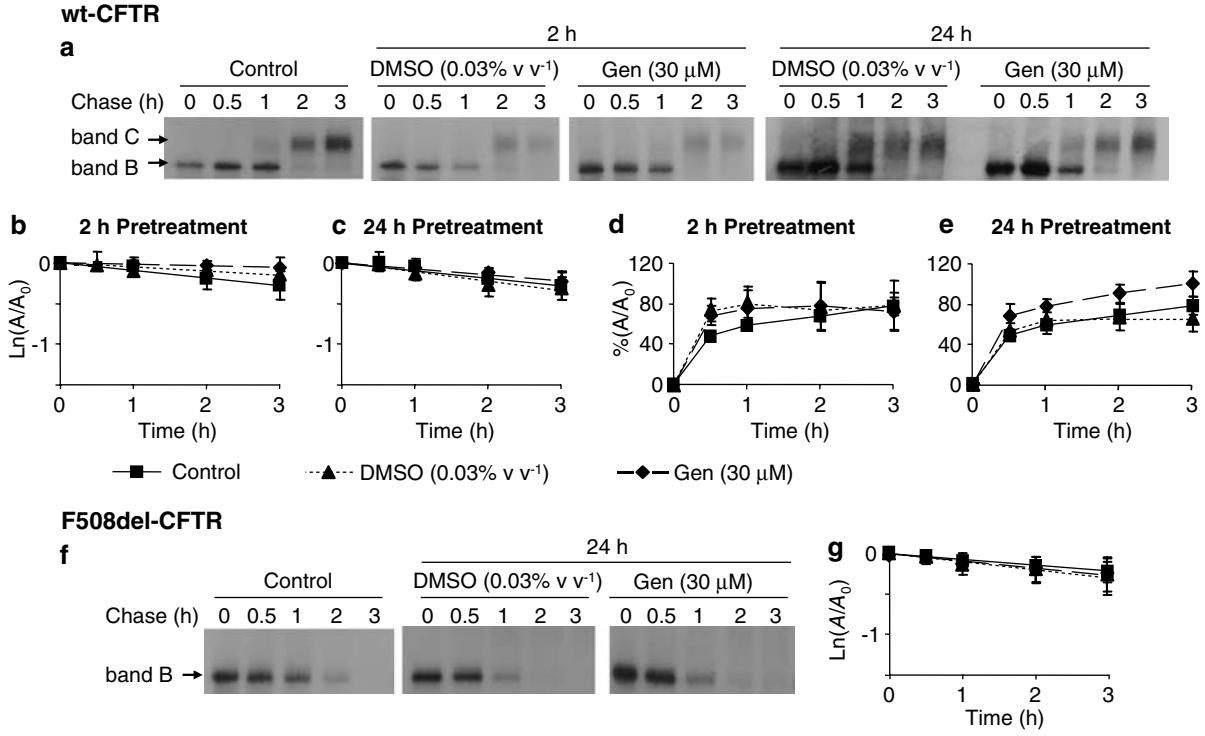

Figure 2 Turnover and processing of cystic fibrosis transmembrane conductance regulator (CFTR) protein following the pretreatment of baby hamster kidney (BHK) cells expressing wild-type and F508del-CFTR with genistein (30 μM). (**a**) Autoradiographs of immunoprecipitated CFTR protein from BHK-wt-CFTR cells pretreated with either dimethyl sulphoxide (DMSO) (0.03% v v^{-1}) or genistein (Gen; 30 μM) for 2 and 24 h at 37 °C. (**f**) Autoradiographs of immunoprecipitated CFTR protein from BHK-F508del-CFTR cells pretreated with either DMSO (0.03% v v^{-1}) or genistein (30 μM) for 24 h at 37 °C. Following drug incubations, BHK-wt-CFTR and BHK-F508del-CFTR cells were radiolabelled and chased for the indicated periods before CFTR was immunoprecipitated and separated by SDS-PAGE followed by fluorography. The positions of bands B and C are indicated by arrows. (**b**, **c**, **g**) Effects of genistein pretreatment on the turnover of band B. Data are presented as the natural logarithm of the amount of band B at a given time of the chase (A) relative to the amount at the beginning of the experiment (A_0). (**d**, **e**) Effects of genistein pretreatment on the efficiency of processing into band C. Data are presented as the percentage of band C detected at a given time of the chase (A) relative to the amount at the beginning of the experiment (A_0). Symbols and error bars are means ± s.e.mean (**b**, **d**, $n=3$; **c**, **e**, **g**, $n=5$). In (**b**, **c**, **g**), the lines are the fit of first-order regressions to the data.

BHK-F508del-CFTR cells were pretreated with these agents for 24 h (Figure 3g). However, these pretreatments altered the efficiency of processing of wild-type CFTR. Figure 3d shows that pretreatment for 2 h with genistein (100 μM) did not have any effect, whereas pretreatment with DMSO decreased processing efficiency, but not significantly ($P>0.05$). The data also suggest that processing efficiency was similar when BHK-wt-CFTR cells were pretreated for 2 h at 37 °C with genistein (30 or 100 μM; Figures 2d and 3d). Conversely, pretreatment for 24 h with genistein (100 μM) altered the maturation of CFTR protein to band C, whereas pretreatment with DMSO did not have any effect (Figure 3e). The band of intermediate mobility between bands B and C (Figure 3a) indicates that some CFTR protein, after post-ER processing, was delivered to the cell membrane where it mediated iodide efflux (Figure 1f). However, the mobility of this band suggests that genistein (100 μM) impairs, albeit not completely, the glycosylation of CFTR in the Golgi apparatus.

Besides acting as a CFTR potentiator, genistein is a potent tyrosine kinase inhibitor (Akiyama and Ogawara, 1991). This raises the possibility that the effects on CFTR processing observed when BHK-wt-CFTR cells are pretreated with genistein (100 μM) for 24 h at 37 °C result from genistein's action as a tyrosine kinase inhibitor rather than its role as a CFTR potentiator. To explore this possibility, we selected for study tyrphostin 47, a tyrosine kinase inhibitor that enhances CFTR-mediated transepithelial Cl^- transport, albeit less efficaciously than genistein (Sears *et al*., 1995). Using identical conditions to those employed with genistein (100 μM), we tested the effects of tyrphostin 47 on CFTR processing.

Figure 4a demonstrates that pretreatment of BHK-wt-CFTR cells with tyrphostin 47 (100 μM) for 24 h at 37 °C led to the appearance of a diffuse band of higher mobility than band C, but lower than that of band B. This band of intermediate mobility between bands B and C was absent in untreated BHK-wt-CFTR cells and also in those pretreated with tyrphostin 47's vehicle DMSO (Figure 4a). Moreover, only the immature form of CFTR was detected in BHK-F508del-CFTR cells pretreated with either tyrphostin 47 (100 μM) or DMSO (0.1% v v^{-1}) for 24 h at 37 °C (Figure 4d). Like the effect of genistein (100 μM) (Figures 3c and g), incubation of BHK-wt-CFTR and BHK-F508del-CFTR cells with tyrphostin 47 (100 μM) for 24 h at 37 °C failed to alter the turnover rate of band B (Figures 4b and e). Similarly, pretreatment of BHK-wt-CFTR cells with either genistein (100 μM) or tyrphostin 47 (100 μM) for 24 h at 37 °C had identical effects on CFTR processing (Figures 3e and 4c). Because both these tyrosine kinase inhibitors generated bands of intermediate mobility

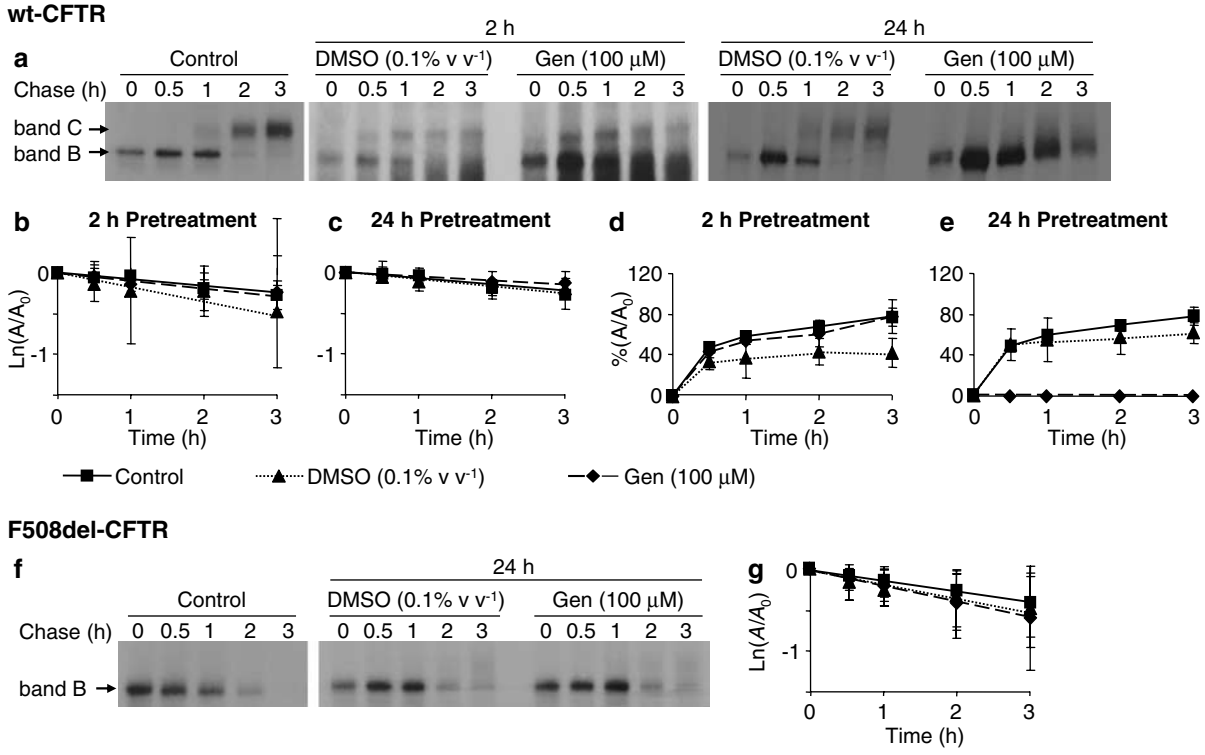

Figure 3 Pretreatment of baby hamster kidney (BHK) cells expressing wild-type and F508del-CFTR with genistein (100 μM) impairs the maturation of wild-type cystic fibrosis transmembrane conductance regulator (CFTR), but is without effect on F508del-CFTR. (**a**) Autoradiographs of immunoprecipitated CFTR protein from BHK-wt-CFTR cells pretreated with either dimethyl sulphoxide (DMSO) (0.1% v v^{-1}) or genistein (Gen; 100 μM) for 2 and 24 h at 37 °C. For comparison, the control autoradiograph is the same as Figure 2a. (**f**) Autoradiographs of immunoprecipitated CFTR protein from BHK-F508del-CFTR cells pretreated with either DMSO (0.1% v v^{-1}) or genistein (100 μM) for 24 h at 37 °C. Following drug incubations, BHK-wt-CFTR and BHK-F508del-CFTR cells were radiolabelled and chased for the indicated periods before CFTR was immunoprecipitated and separated by SDS-PAGE followed by fluorography. The positions of bands B and C are indicated by arrows. (**b**, **c**, **g**) Effects of genistein pretreatment on the turnover of band B. Data are presented as the natural logarithm of the amount of band B at a given time of the chase (A) relative to the amount at the beginning of the experiment (A_0). (**d**, **e**) Effects of genistein pretreatment on the efficiency of processing into band C. Data are presented as the percentage of band C detected at a given time of the chase (A) relative to the amount at the beginning of the experiment (A_0). Symbols and error bars are means ± s.e.mean (**b**, **d**, $n=5$; **c**, **e**, **g**, $n=3$). In (**b**, **c**, **g**), the lines are the fit of first-order regressions to the data.

between bands B and C (Figures 3a and 4a), but tryphostin 47 is a weaker CFTR potentiator than genistein (Sears *et al*., 1995), we suggest that post-ER processing of CFTR might be regulated by tyrosine phosphorylation.

The effects of genistein pretreatment on the localization of CFTR protein

Our biochemical studies demonstrated that genistein pretreatment (2 or 24 h) altered the processing of CFTR protein. This suggests that incubating cells with the drug might alter the distribution of CFTR protein within cells. To test this idea, we performed immunocytochemical studies to localize specifically CFTR protein. Consistent with previous work (for example, Denning *et al*., 1992), in untreated control cells, wild-type CFTR protein was located both intracellularly and at or near the cell membrane, whereas F508del-CFTR was located only intracellularly (Figure 5a). Visual inspection of the images shown in Figures 5b and c suggests that pretreatment of BHK-wt-CFTR and BHK-F508del-CFTR cells with either genistein (30 and 100 μM) or equivalent volumes of DMSO for 2 and 24 h at 37 °C had similar effects on the localization of CFTR protein. For BHK-wt-CFTR cells, pretreatment resulted in the accumulation of more CFTR protein at or near the cell membrane (Figures 5b and c). Moreover, for BHK-F508del-CFTR cells, pretreatment led to a more diffuse distribution of CFTR protein within cells (Figures 5b and c). Thus, these data suggest that the cell surface expression of wild-type CFTR is enhanced following the pretreatment of cells with either genistein or its vehicle DMSO.

The single-channel properties of CFTR are altered by genistein pretreatment

Previous work has demonstrated that genistein interacts with CFTR at multiple sites to modulate channel activity (Wang *et al*., 1998; Lansdell *et al*., 2000). Low micromolar concentrations of genistein potentiate CFTR channel gating by interacting directly with the NBDs (Wang *et al*., 1998; Randak *et al*., 1999). By contrast, elevated concentrations of the drug inhibit the channel both by interfering with NBD-driven channel gating and occluding weakly the channel pore (Lansdell *et al*., 2000). To learn how genistein pretreatment alters the function of wild-type CFTR, we used excised inside-out membrane patches. Disappointingly,

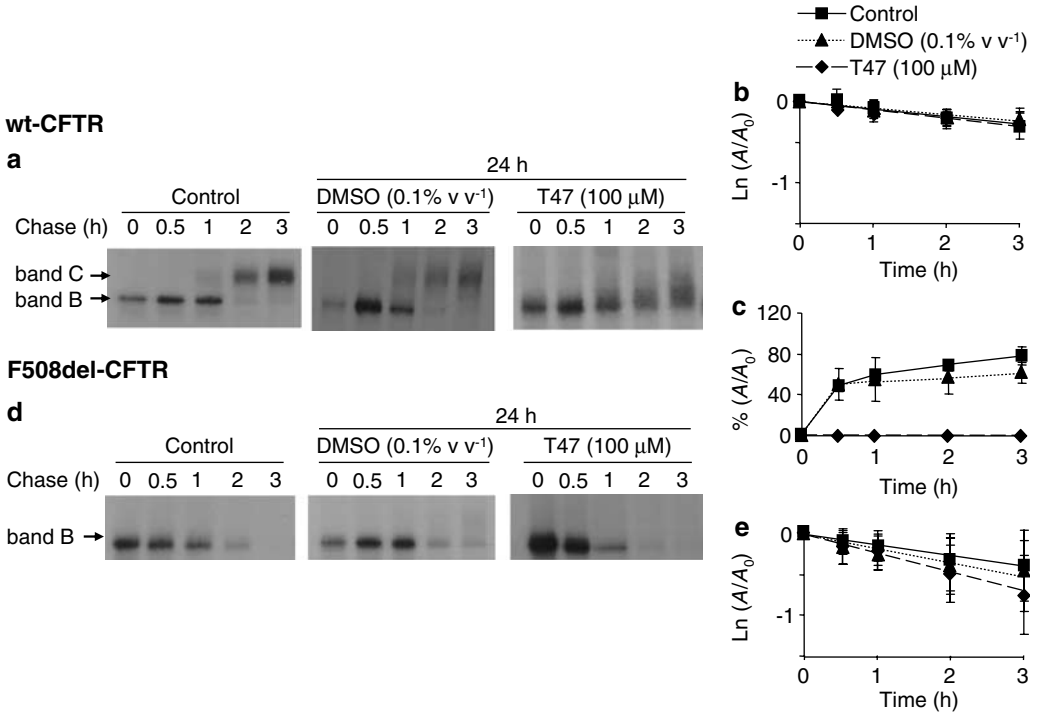

Figure 4 The tyrosine kinase inhibitor tyrphostin 47 impairs the maturation of wild-type cystic fibrosis transmembrane conductance regulator (CFTR). (**a**, **d**) Autoradiographs of immunoprecipitated CFTR protein from BHK-wt-CFTR and BHK-F508del-CFTR cells, respectively, pretreated with either dimethyl sulphoxide (DMSO) (0.1% v v^{-1}) or tyrphostin 47 (T47; 100 μM) for 24 h at 37 °C. Following drug incubations, BHK-wt-CFTR and BHK-F508del-CFTR cells were radiolabelled and chased for the indicated periods before CFTR was immunoprecipitated and separated by SDS-PAGE followed by fluorography. The positions of bands B and C are indicated by arrows. (**b**, **e**) Effects of tyrphostin 47 pretreatment on the turnover of band B. Data are presented as the natural logarithm of the amount of band B at a given time of the chase (*A*) relative to the amount at the beginning of the experiment (A_0). (**c**) Effects of tyrphostin 47 pretreatment on the efficiency of processing into band C. Data are presented as the percentage of band C detected at a given time of the chase (*A*) relative to the amount at the beginning of the experiment (A_0). Symbols and error bars are means ± s.e.mean (control and DMSO pretreatment, $n=5$; tyrphostin 47 pretreatment, $n=3$). In (**b**, **e**), the lines are the fit of first-order regressions to the data.

despite repeated efforts ($n>125$), we were unable to record CFTR Cl^- currents from BHK-wt-CFTR cells pretreated at 37 °C with (i) genistein (30 and 100 μM) for 24 h, (ii) genistein (30 μM) for 2 h and (iii) equivalent volumes of DMSO under similar experimental conditions because it was not possible to form high-resistance seals on pretreated cells.

When we excised inside-out membrane patches from BHK-wt-CFTR cells pretreated with genistein (100 μM) at 37 °C for 2 h, we observed small numbers of active channels in the presence of ATP (1 mM) only and large macroscopic CFTR Cl^- currents after the phosphorylation of CFTR with PKA (75 nM; Figure 6). Visual inspection of channel openings prior to PKA-dependent phosphorylation suggests that genistein pretreatment altered the single-channel properties of CFTR decreasing the current amplitude without altering the duration of channel openings (Figures 6a and b). To quantify the effects of genistein pretreatment, we measured single-channel current amplitude (*i*), MBD, percent time open and frequency of channel gating in the presence of ATP (1 mM) prior to PKA-dependent phosphorylation. We did not calculate interburst interval because the number of active channels in the membrane patch was unknown in the absence of PKA. Figures 6c–f demonstrate that a 2 h pretreatment with genistein (100 μM) depressed single-channel current amplitude ($P<0.05$) without altering MBD, percent time open or frequency of channel gating (all $P>0.05$). We interpret these data to suggest that 2 h pretreatments with genistein (100 μM) inhibit CFTR activity by reducing current flow through open channels.

Noise analysis of the effects of genistein pretreatment on CFTR function

The decrease in single-channel current amplitude caused by pretreating BHK-wt-CFTR cells with genistein (100 μM) resembles the action of several open-channel blockers of the CFTR Cl^- channel (for example, tolbutamide (Venglarik *et al*., 1996) and niflumic acid (Scott-Ward *et al*., 2004)). To investigate the effects of tolbutamide on the kinetics of channel gating, Venglarik *et al*. (1996) used the spectral analysis technique to derive information about gating kinetics from membrane patches containing large numbers of active channels. We therefore adopted a similar strategy to investigate the effects of genistein pretreatment on the kinetics of channel gating following CFTR phosphorylation with PKA. Consistent with previous data (Fischer and Machen, 1994; Venglarik *et al*., 1996), in untreated BHK-wt-CFTR cells, power density spectra of CFTR Cl^- currents were best fitted with two Lorentzian components described by corner frequencies f_{c1} and f_{c2} (Figure 7 and Table 1). The dominant low-frequency Lorentzian component (f_{c1}) corresponds to the bursting pattern of channel gating controlled

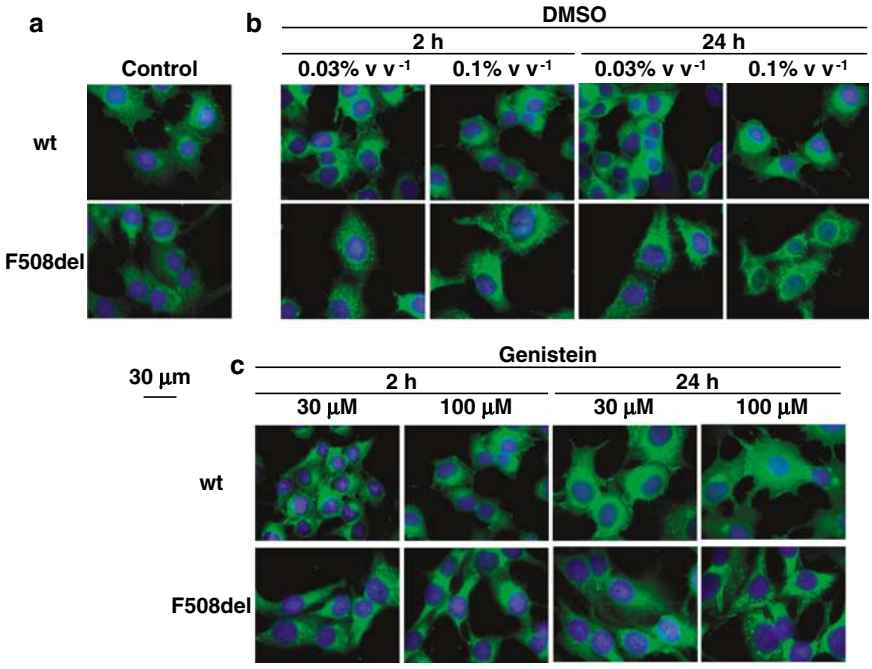

Figure 5 The effects of genistein incubation on the localization of wild-type and F508del-CFTR. Fluorescent immunocytochemical images of BHK-wt-CFTR and BHK-F508del-CFTR cells pretreated with either dimethyl sulphoxide (DMSO) or genistein for 2 and 24 h. (**a**) Untreated controls. (**b**) Cells pretreated with DMSO (0.03 and 0.1% v v^{-1}) for 2 and 24 h. (**c**) Cells pretreated with genistein (30 and 100 μM) for 2 and 24 h. The fluorescein isothiocyanate signal is represented in green and the 4′,6-diamidino-2-phenylindole dihydrochloride nuclear stain in blue. Bar = 30 μm. The results are representative of two independent experiments. No background staining or autofluorescence was observed with untransfected baby hamster kidney (BHK) cells (data not shown).

by the interaction of ATP with the NBDs, whereas the intermediate-frequency Lorentzian component (f_{c2}) with minimal power ($S_{02} = 3.7\%$ of S_{01}; $n = 3$) represents the brief closures that interrupt bursts of channel openings (Fischer and Machen, 1994; Venglarik *et al*., 1994).

Both when CFTR Cl^- currents were acutely treated with genistein (100 μM) and when BHK-wt-CFTR cells were pretreated with genistein (100 μM) prior to channel activation, power density spectra of CFTR Cl^- currents were best fitted with three Lorentzian components described by corner frequencies f_{c1}, f_{c2} and f_{c3} (Figure 7 and Table 1). Like tolbutamide inhibition of CFTR (Venglarik *et al*., 1996), the corner frequency of the genistein-induced component (f_{c3}) was distinguished by its high frequency (Figure 7 and Table 1). Moreover, the corner frequency was the same irrespective of whether CFTR Cl^- currents were acutely treated with genistein or BHK-wt-CFTR cells were pretreated with the drug prior to channel activation (Table 1). We interpret these data to suggest that pretreatment of BHK-wt-CFTR cells with genistein (100 μM) leads to the blockade of CFTR Cl^- channels upon their activation by PKA-dependent phosphorylation.

Discussion and conclusions

The goal of this study was to investigate the long-term effects of the CFTR potentiator genistein on the expression and function of the CFTR Cl^- channel. We demonstrate that pretreatment of BHK-wt-CFTR cells with concentrations of genistein that either potentiate or inhibit the CFTR Cl^- channel alters the processing, cell surface localization and single-channel function of wild-type CFTR.

Genistein is the best-studied CFTR potentiator (for review, see Hwang and Sheppard, 1999; Cai *et al*., 2007). The drug does not open quiescent channels. Instead, it augments the gating of phosphorylated CFTR Cl^- channels by increasing the frequency and duration of channel openings (Wang *et al*., 1998). To explain these effects of genistein, Ai *et al*. (2004) employed the ATP-driven NBD dimerization model of CFTR channel gating (Vergani *et al*., 2003). Ai *et al*. (2004) speculated that genistein accelerates channel opening by promoting NBD dimerization and slows channel closure by stabilizing the NBD1:NBD2 dimer conformation. The authors also proposed that the genistein-binding site might be located at the NBD1:NBD2 dimer interface, a prediction supported by studies of genistein docking to a molecular model of the NBD1:NBD2 dimer (Moran *et al*., 2005). Consistent with functional data (for example, Wang *et al*., 1998), the predicted genistein-binding site in this molecular model is distinct from the two ATP-binding sites of CFTR (Moran *et al*., 2005).

Interestingly, Cui *et al*. (2007) demonstrated that CFTR constructs lacking NBD2 exit the ER, whereas F508del arrests their maturation. These data argue that NBD dimerization is not a prerequisite for CFTR processing. This suggests that CFTR potentiators, such as genistein, which stabilize the NBD1:NBD2 dimer conformation, are unlikely to rescue F508del-CFTR folding. Instead, the data of Cui *et al*. (2007) predict that agents, which bind to NBD1 or enhance the packaging of NBD1 with the membrane-spanning domains

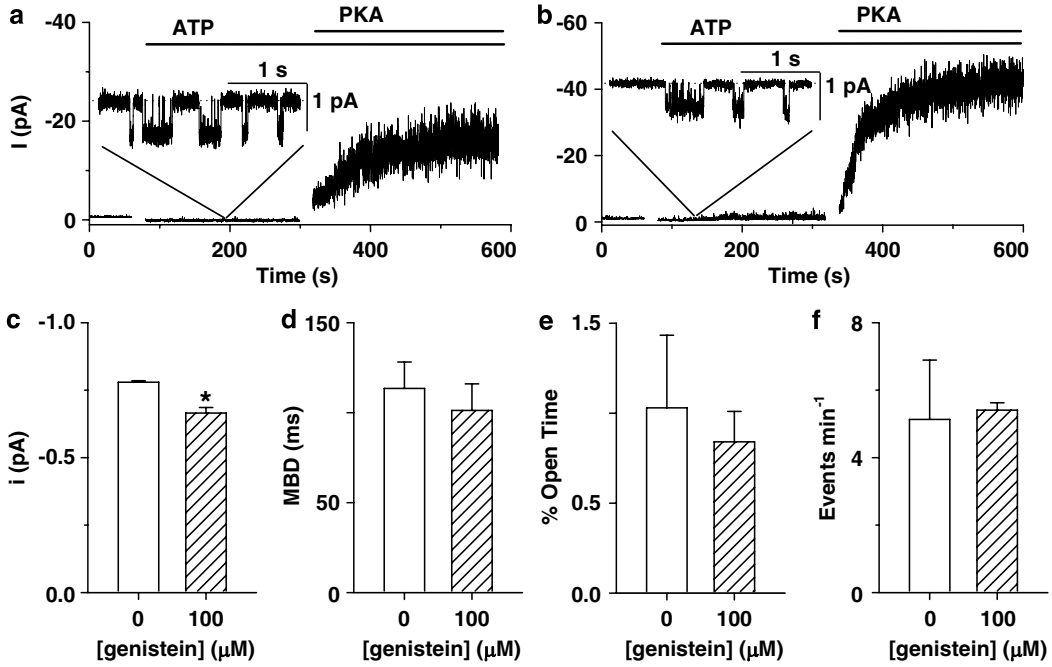

Figure 6 Pretreating BHK-wt-CFTR cells with genistein alters the single-channel properties of cystic fibrosis transmembrane conductance regulator (CFTR). (**a**, **b**) Time courses of CFTR Cl^- currents in excised inside-out membrane patches from BHK-wt-CFTR cells. (**a**) Membrane patch from an untreated control cell. (**b**) Membrane patch from a cell pretreated with genistein (100 μM) for 2 h prior to patch excision. ATP (1 mM) and protein kinase A (PKA) (75 nM) were present in the intracellular solution during the periods indicated by the bars. Voltage was −50 mV and there was a large Cl^- concentration gradient across the membrane ($[Cl^-]_{int} = 147$ mM; $[Cl^-]_{ext} = 10$ mM). For the purpose of illustration, time courses have been inverted so that upward deflections represent inward currents. The inserts show sections of the records prior to CFTR phosphorylation by PKA on an expanded timescale. The dotted lines indicate where channels are closed and downward deflections of the traces correspond to channel openings. (**c**–**f**) Effects of genistein (100 μM) pretreatment on single-channel current amplitude (*i*), mean burst duration (MBD), percent time open and events per minute. Measurements were made in the presence of ATP (1 mM) prior to CFTR phosphorylation with PKA. Abbreviations: 0, untreated; 100, pretreated with genistein (100 μM) for 2 h prior to patch excision. Columns and error bars indicate means + s.e.mean (control: $n = 33$ bursts from five excised membrane patches; genistein: $n = 32$ bursts from three excised membrane patches). The asterisk indicates a value that is significantly different from the control value ($P < 0.05$). Single-channel data were quantified as described in the Materials and methods.

would promote F508del-CFTR folding and hence, rescue its trafficking defect.

In the present study, we demonstrate that pretreatment of BHK-wt-CFTR cells with genistein alters CFTR-mediated iodide efflux. These effects of genistein might reflect the direct interaction of the drug with CFTR. Alternatively, they might result from the actions of genistein on proteins with which CFTR interacts during its biogenesis within the cell and function at the cell surface. As discussed below, the effects of genistein (100 μM) pretreatment on the single-channel behaviour of CFTR argue that the drug interacts directly with the CFTR Cl^- channel. Thus, there are similarities between the acute and chronic actions of the drug on CFTR. However, our data also suggest that genistein pretreatment might alter CFTR expression and function by other mechanisms. First, genistein is a potent inhibitor of tyrosine kinases (Akiyama and Ogawara, 1991). This suggest that pretreating BHK-wt-CFTR cells with the drug might perturb the tyrosine phosphorylation status of proteins within these cells and hence the trafficking and/or function of CFTR. Consistent with this idea, prolonged incubation of BHK-wt-CFTR cells with high concentrations of genistein or another inhibitor of tyrosine kinases, tyrphostin 47, generated a band of intermediate mobility between bands B and C. The data suggest that both these agents impair, albeit not completely, CFTR processing. Because BHK-wt-CFTR cells pretreated with genistein (100 μM) for 24 h at 37 °C generated an efflux of iodide, this intermediate form of CFTR protein is probably delivered to the cell surface where it exhibits some function. Alternatively, biochemically undetectable amounts of fully processed protein might account for the observed efflux. Thus, the late stages of CFTR processing appear to be directly or indirectly regulated by tyrosine phosphorylation.

Second, genistein might interact with lipids to modulate the activity of CFTR, itself or CFTR-interacting proteins. Consistent with this possibility, Hwang *et al*. (2003) demonstrated that genistein modulates the behaviour of gramicidin A channels by altering the mechanical properties of the phospholipid bilayer in which the channel is imbedded.

Third, it is feasible that genistein pretreatment might have untoward effects on cell proliferation and viability. In support of this idea, Lim *et al*. (2004) demonstrated that concentrations of genistein exceeding 5 μM caused a dose-dependent loss of the viability of IB3-1 cells ((F508del/W1282X) bronchial epithelial cells) after 72 h of treatment, while Li *et al*. (2004) reported similar results with Madin Darby canine kidney epithelial cells using genistein (100 μM). However, we observed that pretreatment of BHK-wt-CFTR cells with genistein (30 and 100 μM) or DMSO (0.03 and 0.1% v v^{-1}) for 24 h at 37 °C did not have any effect

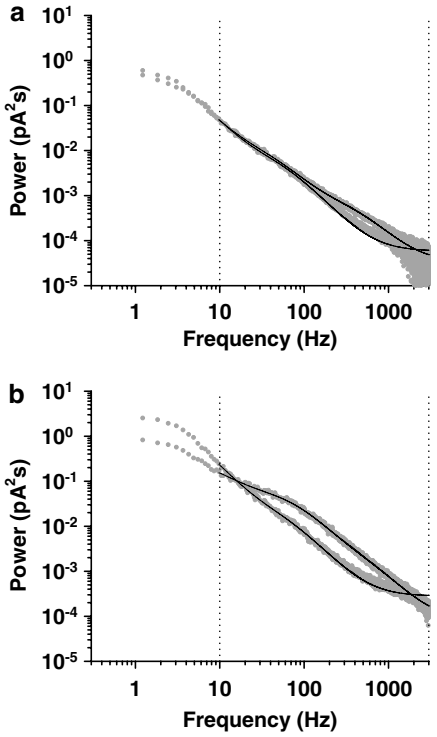

Figure 7 Spectral analysis of genistein inhibition of the CFTR Cl^- channel. (**a**) Power density spectra calculated from data recorded under control conditions (ATP, 1 mM and protein kinase A (PKA), 75 nM) and after the addition of genistein (100 μM) to the intracellular solution of an excised membrane patch from a BHK-wt-CFTR cell. Voltage was −50 mV and there was a large Cl^- concentration gradient across the membrane ($[Cl^-]_{int} = 147$ mM; $[Cl^-]_{ext} = 10$ mM). The solid lines represent the fit of two and three Lorentzian components to the control and genistein (100 μM) data, respectively. Control spectra displayed corner frequencies (f_{c1} and f_{c2}) of 1.23 and 57.08 Hz with maximum variances per unit frequency (S_{01} and S_{02}) of 2.75 and 6.2×10^{-3} pA^2 s. In the presence of genistein (100 μM), corner frequencies (f_{c1}, f_{c2} and f_{c3}) were 2.93, 43.80 and 457.04 Hz with maximum variances (S_{01}, S_{02} and S_{03}) of 0.48, 8.3×10^{-3} and 5.7×10^{-4} pA^2 s. (**b**) Power density spectra calculated using data from a membrane patch excised from a BHK-wt-CFTR cell pretreated with genistein (100 μM) for 2 h prior to patch excision. The solid lines represent the fit of three Lorentzian components to the genistein (100 μM) data. For comparison, control spectra from (**a**) and the fit of two Lorentzian components to these data are shown. The spectra from the genistein (100 μM) pretreated cell had corner frequencies (f_{c1}, f_{c2} and f_{c3}) of 7.76, 75.98 and 633.04 Hz with maximum variances (S_{01}, S_{02} and S_{03}) of 0.26, 5.3×10^{-2} and 1.3×10^{-3} pA^2 s. Power density spectra were constructed as described in the Materials and methods.

on cell viability ($n = 4$–8; $P > 0.05$; data not shown), suggesting that the genistein pretreatment-induced changes in iodide efflux are not a consequence of cytotoxic effects of the drug.

By following the fate of radiolabelled CFTR protein, we evaluated the effects of genistein pretreatment on CFTR maturation. Our data reveal that genistein pretreatment did not have any effect on the stability of immature CFTR protein in cells expressing wild-type- or F508del-CFTR. However in cells expressing wild-type-CFTR, genistein consistently increased, albeit slightly, the efficiency with which immature CFTR protein is processed into its mature form. They also suggest that pretreatment with genistein (100 μM) has widespread effects on the cellular pathways responsible for CFTR processing and trafficking. While the identity of the CFTR-interacting proteins, which might be affected by genistein (100 μM), are unknown, Lim *et al*. (2004) demonstrated that pretreatment of IB3-1 cells with genistein (5 μM) for 48 h at 37 °C did not have any effect on the chaperones Hsp70, Hsc70, calreticulin and calnexin.

Lehrich *et al*. (1998) found that acute treatment of shark rectal glands with genistein (100 μM) promoted the trafficking of CFTR protein to the apical membrane of gland-lining epithelial cells, while Lim *et al*. (2004) showed that long-term treatment of IB3-1 cells with genistein (5 μM) led to a dispersed distribution of F508del-CFTR protein throughout the cytoplasm. Consistent with these data, our immunocytochemical images demonstrate that all genistein pretreatments tested led to the accumulation of wild-type CFTR protein at or near the cell membrane and a diffuse distribution of F508del-CFTR protein within cells. However, our biochemical data reveal that different concentrations of genistein have disparate effects on protein maturation. A likely explanation for this apparent incongruence is the pool of CFTR protein studied. Pulse-chase experiments only detect radiolabelled protein generated after cells are pretreated with genistein. By contrast, immunocytochemistry visualizes all CFTR protein in the cell, including protein produced before cells are treated with genistein.

Elevated concentrations of genistein inhibits CFTR by two mechanisms: allosteric and open-channel block (Lansdell *et al*., 2000; Cai *et al*., 2007). Here, we show that following a 2 h pretreatment of BHK-wt-CFTR cells with genistein (100 μM), CFTR Cl^- channels exhibit characteristics of open-channel block (Hille, 2001; Venglarik *et al*., 1996; Scott-Ward

Table 1 Effect of genistein on corner frequencies derived from spectral analysis of CFTR Cl^- channels

Condition	f_{c1} *(Hz)*	f_{c2} *(Hz)*	f_{c3} *(Hz)*	n
Control	4.05 ± 2.10	74.60 ± 6.23	—	5
Genistein (100 μM) acute treatment	3.64 ± 1.01	58.14 ± 4.74	570.54 ± 45.80	5
Genistein (100 μM) 2 h pre-incubation	4.25 ± 2.19	67.56 ± 6.28	539.40 ± 104.66	3

Abbreviation: CFTR, cystic fibrosis transmembrane conductance regulator.
Corner frequencies were measured using the indicated conditions. Under control conditions, the behaviour of CFTR Cl^- channels is described by the sum of two Lorentzian components (f_{c1} and f_{c2}). Either when excised membrane patches are treated directly with genistein (100 μM) or when BHK-wt-CFTR cells are pretreated with genistein (100 μM) prior to membrane patch excision, an additional high-frequency Lorentzian component (f_{c3}) is required to fit the data. Values are means ± s.e.mean of *n* observations. Using a one-way ANOVA, values of f_{c1} and f_{c2} did not change significantly between the conditions tested ($P > 0.1$), neither did those of f_{c3} between the two conditions tested ($P > 0.75$). Measurements were made in the presence of the catalytic subunit of protein kinase A (PKA) (75 nM) and ATP (1 mM) in the intracellular solution. Voltage was −50 mV and there was a large Cl^- concentration gradient across the membrane ($[Cl^-]_{int} = 147$ mM; $[Cl^-]_{ext} = 10$ mM).

et al., 2004). For two reasons, this result was unexpected. First, genistein is a low-affinity open-channel blocker of CFTR (K_d(0 mV) ~1800 μM; Lansdell *et al*., 2000). Second, both drug-treated cells and excised membrane patches were thoroughly washed prior to the study. Under these conditions, we would expect genistein to be absent from the solutions bathing the intra- and extracellular sides of excised membrane patches. This suggests that the most likely source of genistein is the cell membrane, based on the data of Hwang *et al*. (2003) and the hydrophobic nature of this flavonoid. This raises the interesting question of how genistein within the lipid bilayer causes open-channel block of CFTR. Genistein might alter the mechanical properties of the cell membrane or access its drug-binding site within the CFTR pore directly from the cell membrane. However, the simplest interpretation of the data (Lansdell *et al*., 2000; present study) is that genistein within the cell membrane enters the intracellular solution where its anionic form is swept deep inside the CFTR pore by the prevailing electrochemical gradient. When genistein binds, its large size occludes the CFTR pore preventing Cl^- permeation. Alternatively, during drug pretreatment, genistein entering the cell might saturate the environment and bind to CFTR protein en route to the cell surface. This explanation is consistent with the observed intermediate mobility of partially processed CFTR protein when BHK-wt-CFTR cells were pretreated with genistein (100 μM) for 24 h at 37 °C.

Because of the difficulties in forming high-resistance seals, we did not determine the effects on CFTR function of incubating BHK-wt-CFTR cells with genistein (30 μM). Based on the data of Hwang *et al*. (2003), our failure to form high-resistance seals on genistein pretreated cells might result from the drug's effects on the mechanical properties of the lipid bilayer. Interestingly, Bulteau-Pignoux *et al*. (2002) demonstrated that brief (~2 min) exposure of CHO cells expressing wild-type CFTR to genistein (25 μM) prior to forskolin (2.5 μM) slowed CFTR activation and attenuated markedly current magnitude. The authors interpreted their data to suggest that the binding of genistein to the NBDs might impede regulatory domain phosphorylation by PKA. However, the interaction of genistein with the cell membrane (Hwang *et al*., 2003) might influence directly either CFTR channel gating or current flow through the channel.

DMSO is widely used as a vehicle for CFTR modulators. When used acutely, it does not have any effect on CFTR function (for example, Sheppard and Robinson, 1997). By contrast, prolonged exposure of cells to DMSO influences the expression and localization of CFTR in IB3-1 and BHK cells (Lim *et al*., 2004; present study). However, our data argue that the effects of DMSO do not explain those of genistein on CFTR expression and function. Previous work has shown that DMSO acts as an epithelial-differentiating agent, which enhances tight junction organization (Bebök *et al*., 1998). Bebök *et al*. (1998) exploited this property of DMSO to promote the delivery of F508del-CFTR to the apical membrane of LLC-PK_1 renal epithelia using high doses for prolonged periods. Interestingly, DMSO did not have any effect on F508del-CFTR expressed in mouse L cells, a fibroblast cell line, suggesting that the effects of DMSO on F508del-CFTR are cell-type specific (Bebök *et al*., 1998). Taken together, these data are an important reminder that studies of CFTR correctors and potentiators require judicious selection of cell lines and rigorous controls.

In conclusion, we demonstrated that genistein modulates the biosynthesis, localization and function of the CFTR Cl^- channel. All concentrations of the drug tested promoted the accumulation of CFTR protein at the cell surface. However, the effects of genistein were concentration dependent: low micromolar concentrations augmented protein maturation. By contrast, higher concentrations impaired the full maturation of CFTR protein and inhibited channel activity by attenuating current flow through open channels. As a result, when we evaluated CFTR function in intact cells using the iodide efflux technique, we found that low concentrations of genistein augmented CFTR-mediated iodide efflux, whereas high concentrations had the converse effect. These data highlight the importance of a multidisciplinary approach when investigating how CFTR potentiators rescue CF mutants. They also demonstrate the potential of CFTR potentiators to influence CFTR activity by mechanisms distinct from their effects on channel gating. Significantly, some CFTR potentiators (for example, VRT-532; Van Goor *et al*., 2006; Wang *et al*., 2006) have been identified that act as pharmacological chaperones (Bernier *et al*., 2004), rescuing the cell surface expression of F508del-CFTR and enhancing its Cl^- channel function.

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Conflict of interest

The authors state no conflict of interest.

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