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Effect of genistein on native epithelial tissue from normal individuals and CF patients and on ion channels expressed in *Xenopus* oocytes

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1 The flavonoid genistein has been shown to activate a Cl^- conductance in various cell types expressing CFTR. We examined if similar effects can be observed when genistein is applied to native *ex vivo* tissues from human respiratory tract and rectum. We further compared the effects when genistein was applied to oocytes of *Xenopus laevis* expressing CFTR.

2 In oocytes, both wtCFTR and ΔF508-CFTR were activated by genistein while both cyclic AMP (K_vLQT1) and Ca^{2+} (SK4) activated K^+ channels were inhibited at high concentrations of genistein.

3 Biopsies from nasal polyps and rectal mucosa were obtained from normal individuals (non-CF) and CF patients and in the presence of amiloride (10 μmol l^{-1}; mucosal side) the effects of genistein were assessed using a perfused Ussing chamber. In non-CF airway epithelia, genistein (50 μmol l^{-1}; mucosal side) increased lumen negative I_{sc} but had no additional effects on tissues pre-stimulated with IBMX and forskolin (100 μmol l^{-1} and 1 μmol l^{-1}; both sides).

4 In non-CF rectal biopsies, in the presence of amiloride (10 μmol l^{-1}; mucosal side) and indomethacin (10 μmol l^{-1}; basolateral side), genistein increased lumen negative I_{sc} and enabled cholinergic (carbachol; CCH, 100 μmol l^{-1}; basolateral side) stimulation of Cl^- secretion indicating activation of luminal CFTR Cl^- channels. However, after stimulation with IBMX/forskolin, genistein induced opposite effects and significantly inhibited CCH activated I_{sc}. In CF airway and intestinal tissues genistein failed to induce Cl^- secretion.

5 Thus, genistein is able to activate luminal CFTR Cl^- conductance in non-CF tissues and mutant CFTR in oocytes. However, additional inhibitory effects on basolateral K^+ conductance and missing effects in native CF tissues do not support the use for pharmacological intervention in CF. *British Journal of Pharmacology* (2000) **130,** 1884–1892

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Abbreviations: cyclic AMP, cyclic adenosinemonophosphate; CCH, carbachol; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; IBMX, isobutylmethylxanthine; I_{sc}, equivalent short circuit current; K_vLQT1, voltage dependent delayed activated K^+ channel; R_{te}, transepithelial resistance; SK4, Ca^{2+} activated K^+ channel; V_{te}, transepithelial voltage

Introduction

Cystic fibrosis is caused by any of the more than 800 mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), the protein that forms a luminal cyclic AMP regulated Cl^- conductance in epithelial cells (Sheppard & Welsh, 1999; Kunzelmann, 1999). Different mutations affect CFTR function in different ways, however, as a common result the number of active CFTR Cl^- channels and thus the apical Cl^- conductance is reduced in CF (Tsui, 1997). An essential treatment would therefore target the primary defect of mutant CFTR. In that respect, several drugs have been evaluated for their positive effects on expression of mutant CFTR and boosting residual Cl^- channel activity of mutant CFTR. Those drugs comprise inhibitors of phosphodiesterases, xanthin derivatives, bromotetramisole, Benzo[c]quinolizinium compounds and drugs that were shown to increase CFTR expression like phenylbutyrate or glycerol (Becq *et al*., 1999; Schultz *et al*., 1999; Kunzelmann, 1999; Kunzelmann & Nitschke, 2000). In addition flavonoids which are common food ingredients were demonstrated to be potential activators of CFTR (Illek *et al*., 1999; Schultz *et al*., 1999; Illek & Fischer, 1998). Because of their abundance and their potential low risk, these drugs have been suggested to be particularly helpful in the treatment of cystic fibrosis. Genistein and other flavonoids have been demonstrated to very effectively activate CFTR Cl^- conductance once CFTR has been pre-stimulated *via* the cyclic AMP dependent pathway. Thus, the effects of genistein have been examined under various experimental conditions and have been tested in different cell types (Sears *et al*., 1995; Illek & Fischer, 1998; Illek *et al*., 1995).

Despite the relatively large number of studies, there are only a few reports on the effects of genistein on native human epithelium and to our knowledge there is no present study on either native CF airways or intestinal epithelium (Illek & Fischer, 1998). In the present study we therefore addressed the question of the efficacy of genistein mediated activation of Cl^- secretion in native human epithelial tissues that are highly differentiated. To that end we examined *ex vivo* biopsies from human airways and rectum by means of a modified perfused Ussing chamber and compared the effects of genistein on both

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tissues obtained from normal individuals and CF patients. The results obtained in the present experiments and additional experiments in CFTR expressing *Xenopus* oocytes do confirm stimulatory effects of genistein on wtCFTR and ΔF508-CFTR in oocytes and activation of Cl^- secretion in non-CF tissues. However, additional inhibitory effects on Cl^- secretion in non-CF and missing stimulatory effects on CF biopsies do not support the use of this compound for treatment of cystic fibrosis.

Methods

Patients

Freshly excised nasal polyps were obtained from 22 non-CF individuals (mean age: 30.7±3.8 years, range 3–70 years) and 11 CF patients (11±1.9 years, range 4–27 years). Eight CF patients presenting with nasal polyps were tested for six common mutations: ΔF508, R553X, N1303K, G542X, G551D and R347P. The following genotypes were identified: ΔF508/ΔF508 ($n=3$); ΔF508/G551D ($n=1$); ΔF508/−($n=2$); −/− ($n=2$) (− = mutation not identified). In three CF patients where no genetic testing was performed, the diagnosis of CF was established on clinical criteria and elevated sweat Cl^- concentration (Rosenstein & Cutting, 1998). Rectal mucosa tissue biopsies were obtained from 25 non-CF individuals (mean age: 23.9±4.5 years, range: 7 months to 67 years) and 12 CF patients (mean age: 19.5±3.7 years, range: 5 months to 37 years). In all CF patients from whom rectal biopsies were studied DNA analysis was carried out for the following CFTR mutations: ΔF508; R117H and S108F in exon 4; R347P, R347H, I336K and T338I in exon 7; S549N, G551D, R553X, G542X, Q552X, 1717-1 G→A in exon 11; W1282X and 3905insT in exon 20; N1303K in exon 21 and 3849+10kB C→T in intron 19. Screening for these mutations identified the genotypes as follows: ΔF508/ΔF508 ($n=2$); ΔF508/R553X ($n=3$); ΔF508/N1303K ($n=1$); ΔF508/3905insT ($n=1$); ΔF508/−($n=5$) (− = mutation not identified). All CF subjects fulfilled the diagnostic criteria of CF including elevated sweat tests (Rosenstein & Cutting, 1998). Small superficial tissue biopsies were obtained by rectoscopy and forceps biopsy performed at the University Children's Hospital Freiburg. The study was approved by the ethical committee and the patients had given their written informed consent. For children under the age of 18 years parents obtained detailed information and gave their signed informed consent.

Ussing chamber experiments

Nasal polyps and rectal tissue biopsies were immediately stored in an ice cold buffer solution of the following composition (mmol l^{-1}): NaCl 127, KCl 5, D-glucose 5, $MgCl_2$ 1, Na-pyruvate 5, HEPES 10, $CaCl_2$ 1.25 and albumin (10 g l^{-1}). A thin layer of respiratory epithelium was dissected from the stroma of nasal polyps. Nasal and intestinal epithelial tissues were mounted into a perfused micro-Ussing chamber with a circular aperture of 0.95 mm^2 as described previously (Mall *et al*., 1998b). In brief the luminal and basolateral sides of the epithelium were perfused continuously at a rate of 10 ml min^{-1} (chamber volume 1 ml) allowing for the paired examination of the effects of genistein in the presence or absence of cyclic AMP stimulation. The bath solution was prepared freshly on the day of the experiment and had the following composition (mmol l^{-1}): NaCl 145, KH_2PO_4 0.4, K_2HPO_4 1.6, D-glucose 5, $MgCl_2$ 1, Ca-gluconate 1.3, and pH was adjusted to 7.4 at room temperature (22°C) using NaOH (1 mol l^{-1}). Bath solutions were heated by a water jacket to 37°C. Experiments were carried out under open circuit conditions. Transepithelial resistance (R_{te}) was determined by applying short (1 s) current pulses ($\Delta I = 0.5\ \mu A$) and the corresponding changes in V_{te} (ΔV_{te}) and basal V_{te} were recorded continuously. Values for the transepithelial voltage (V_{te}) were referred to the serosal side of the epithelium. Resistance of the empty chamber was 9.5 Ωcm^2. Voltage deflections obtained under conditions without the mucosa present (ΔV_{te}) were subtracted from those obtained in the presence of the tissues. R_{te} was calculated according to Ohm's law ($R_{te} = (\Delta V_{te} - \Delta V_{te})/\Delta I$). The equivalent short circuit current (I_{sc}) was determined from V_{te} and R_{te}, i.e. $I_{sc} = V_{te} / R_{te}$. Tissues were allowed to equilibrate for 60 min. All experiments were carried out in a strictly paired fashion. Between each step genistein was washed out for 30 min. The whole protocol typically took 4–5 h. Tissue preparations were only accepted when R_{te} exceeded the resistance of the empty chamber by a factor of two and when stable recordings of V_{te} and R_{te} were obtained over the whole time frame of the experiment. Furthermore tissue viability was assessed at the end of each experiment by testing the effect of amiloride (10 μmol l^{-1}, mucosal side) in nasal tissues and CCH (100 μmol l^{-1}, basolateral side) in rectal biopsies. The effects of genistein were assessed in non-CF and CF nasal and rectal epithelia and on ion channels expressed in *Xenopus* oocytes. Concentration response curves for the effect of genistein (1, 10, 50, 100 μmol l^{-1}; mucosal side) were constructed from the mean values. The data were fitted with a fit routine using the equation: $I = I_{max} / [1 + (C_{50}/C_n)^n]$. The EC_{50} values were obtained during the fit routine.

Preparation of oocytes and cRNA, microinjection and double electrode voltage clamp

Isolation and microinjection of oocytes have been described in a previous report (Mall *et al*., 1996). In brief, after isolation from 3–4 different adult *Xenopus laevis* female frogs, oocytes were dispersed and defolliculated by a 0.5 h-treatment with collagenase (type A, Boehringer, Germany). Subsequently, oocytes were rinsed and kept in ND96-buffer (in mmol l^{-1}): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-pyruvate 2.5, pH 7.55, supplemented with theophylline (0.5 mmol l^{-1}) and gentamycin (5 mg l^{-1}) at 18°C. Theophylline was included into the media in order to suppress maturation of oocytes (Schorderet-Slatkine & Baulieu, 1982). cDNAs for wtCFTR and ΔF508-CFTR (Hipper *et al*., 1995), rK_vLQT1, hSK4 (generous gift of W.J. Joiner, New Haven, U.S.A. (Warth *et al*., 1999)) and the $P2Y_2$ receptor (kindly provided by W.R. Rice, Cincinnati, U.S.A.) were linearized by either *Hpa*I, *Not*I or *Kpn*I and cRNAs were *in vitro* transcribed using Sp6 or T7 polymerases and a 5′ cap (mCAP mRNA capping kit, Stratagene). Oocytes were injected with 10 ng of each cRNA and water injected oocytes of identical batches served as controls (PV830 pico pump, WPI, Germany). Three days after injection oocytes were impaled with two electrodes (Clark instruments) which had resistances of 1 MΩ when filled with 2.7 mol l^{-1} KCl. A flowing (2.7 mol l^{-1}) KCl electrode served as bath reference. The bath solution (ND96) had the following composition (mmol l^{-1}): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-pyruvate 2.5 and pH was adjusted to pH 7.55. Membrane currents were measured by voltage clamping of the oocytes (OOC-1 amplifier, WPI, Germany). Data were collected continuously on a computer hard disc at a sample frequency of 1000 Hz and were analysed using the programs

chart and scope (McLab, AD-Instruments, Macintosh). During experiments the bath was continuously perfused at a rate of 5–10 ml min^{-1}. All experiments were conducted at room temperature (22°C).

Compounds and statistics

Amiloride, indomethacin, genistein and IBMX were all obtained from Sigma (Deisenhofen, Germany). Forskolin was obtained from Hoechst (Frankfurt/Main, Germany). All used chemicals were of highest grade of purity available. Amiloride, indomethacin, genistein and IBMX were prepared as $\geqslant 1000$ fold stock solutions in dimethyl sulphoxide (DMSO). Forskolin was prepared as a 10,000 fold stock solution in methanol. The vehicles alone (DMSO or methanol) at the final concentration had no effect on V_{te}, R_{te} or I_{sc} in native human nasal or rectal tissues or on *Xenopus* oocytes.

From some individuals transepithelial measurements were performed on more than one tissue sample. When multiple samples were studied by the same protocol data were averaged to obtain a single mean value for each individual subject. Data are shown as original recordings or as mean ± s.e.mean for transepithelial measurements (n = number of subjects) and double voltage clamp studies on *Xenopus* oocytes (n = number of experiments). Statistical analysis was performed using paired Student's t-test. Data obtained from CF and non-CF tissues were compared by the unpaired Student's t-test. If not stated otherwise, P values <0.05 were accepted to indicate statistical significance.

Results

Effect of genistein on human non-CF and CF nasal epithelia

Following equilibration in the Ussing chamber nasal tissues from non-CF individuals had a lumen negative I_{sc} of -37.4 ± 5.8 μA cm^{-2}. V_{te} was -0.5 ± 0.1 mV and R_{te} was 18.2 ± 2.6 Ωcm^2 ($n = 22$). In nasal epithelia obtained from CF patients I_{sc} and V_{te} were significantly increased to -103.5 ± 25.2 μA cm^{-2} and -1.4 ± 0.5 mV, compared to non-CF and R_{te} was slightly reduced to 14.6 ± 1.8 Ωcm^2 ($n = 11$). The contribution of amiloride-sensitive Na^+ channels (ENaC) and cyclic AMP-dependent CFTR Cl^- channels to ion transport in non-CF and CF airway epithelia was assessed by adding (i) amiloride (10 μmol l^{-1}; mucosal side) and (ii) IBMX and forskolin (100 μmol l^{-1} and 1 μmol l^{-1}; both sides). As expected from previous studies (Boucher *et al*., 1988; Mall *et al*., 1998a; 1999) the amiloride-sensitive I_{sc} in nasal tissues from CF patients ($\Delta I_{sc} = 92.2 \pm 21.4$ μA cm^{-2}; $n = 11$) was significantly increased ($P < 0.0003$) compared to non-CF ($\Delta I_{sc} = 27.6 \pm 5.3$ μA cm^{-2}; $n = 22$). Stimulation with IBMX/forskolin significantly enhanced I_{sc} only in nasal epithelia from non-CF ($\Delta I_{sc} = -18.5 \pm 4.6$ μA cm^{-2}; $n = 22$) but not from CF ($\Delta I_{sc} = -4.7 \pm 2.8$ μA cm^{-2}; $n = 11$) subjects (Figure 1). To examine if the increase of lumen negative I_{sc} in tissues from non-CF individuals was caused by Cl^- secretion, bumetanide (50 μmol l^{-1}; basolateral side) was added in the presence of cyclic AMP dependent stimulation. In this series IBMX/forskolin significantly increased I_{sc} from -29.7 ± 9.8 μA cm^{-2} to -64.2 ± 14.3 μA cm^{-2} ($n = 5$). Addition of bumetanide abolished cyclic AMP activated I_{sc} completely and significantly reduced lumen negative I_{sc} down to -20.5 ± 3.2 μA cm^{-2} ($n = 5$) indicating activation of Cl^- secretion. After blocking ENaC by amiloride, effects of genistein (50 μmol l^{-1}; mucosal side) were assessed in paired experiments in both absence and presence of cyclic AMP (IBMX/forskolin) dependent stimulation (Figures 1B, C and 2B). In non-CF, under basal conditions, genistein enhanced lumen negative V_{te} and significantly

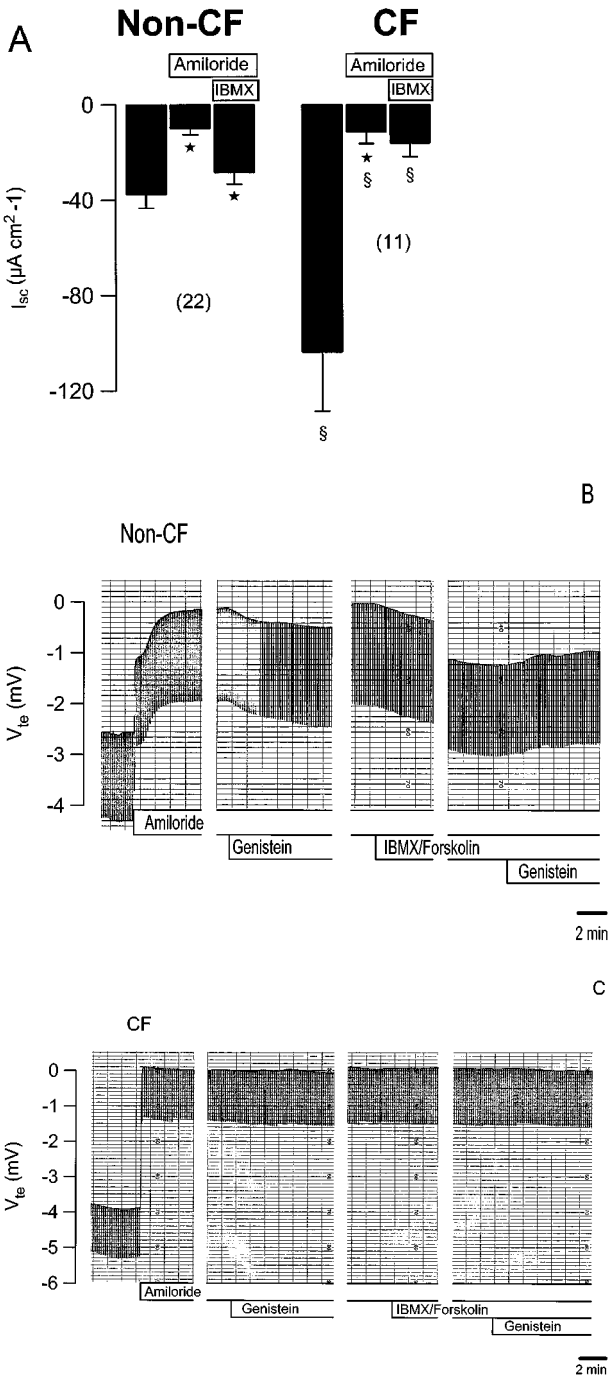


Figure 1 (A) Summary of short circuit currents (I_{sc}) under resting conditions, after blocking epithelial Na^+ channels by amiloride (10 μmol l^{-1}; mucosal side) and after activation of ion transport by IBMX and forskolin (100 μmol l^{-1} and 1 μmol l^{-1}; both sides) in human nasal epithelia from non-CF and CF individuals. *Indicates statistical significance for the effects of amiloride and IBMX/forskolin (paired t-test). §Indicate statistical significance for the basal I_{sc} and the effects of amiloride and IBMX/forskolin when compared to experiments obtained from non-CF tissues (unpaired t-test). (Number of subjects). Effect of genistein (50 μmol l^{-1}; mucosal side) on transepithelial voltage (V_{te}) in (B) non-CF and (C) CF human nasal tissues. Epithelial Na^+ conductance was blocked by amiloride and effects of genistein were examined in both absence and presence of IBMX and forskolin. R_{te} was determined continuously from the V_{te} downward deflections obtained by pulsed current injection.

increased I_{sc} in a dose dependent fashion with an EC_{50} of approximately 3.8 μmol l^{-1} (Figure 2A). Maximal responses of genistein were observed at a concentration of 50 μmol l^{-1} with an absolute magnitude of -15.0 ± 3.2 μA cm^{-2} ($n=12$). As shown in Figure 2A, genistein inhibited I_{sc} at concentrations higher than 50 μmol l^{-1}. Submaximal activation of CFTR by 0.1 μmol l^{-1} forskolin (both sides) increased I_{sc} slightly but significantly. Under these conditions, 50 μmol l^{-1} genistein further increased I_{sc} Genistein had no additional effect on I_{sc} after maximal stimulation of CFTR by 100 μmol l^{-1} IBMX and 1 μmol l^{-1} forskolin (Figure 2B). In CF nasal epithelia, no significant changes of I_{sc} were observed upon either submaximal or maximal stimulation of the cyclic AMP dependent pathway (Figure 2B). Furthermore genistein (50 μmol l^{-1}) failed to induce Cl^- secretion in CF tissues under any conditions as shown in Figures 1C and 2B.

Effect of genistein on human non-CF and CF rectal epithelia

Apart from the airways, CFTR is expressed at high levels in colon and rectum and thus Cl^- secretion was found defective in the intestinal tract of CF patients. Therefore, we examined the effects of genistein in rectal biopsies from non-CF and CF subjects. Under resting conditions rectal tissues from non-CF individuals had a lumen negative I_{sc} of -37.4 ± 5.3 μA cm^{-2}. V_{te} was -0.9 ± 0.2 mV and R_{te} was 19.8 ± 3.0 Ωcm^2 ($n=12$). In rectal biopsies from CF patients I_{sc} was slightly but not significantly decreased to -26.0 ± 10.6 μA cm^{-2} and V_{te} was -1.5 ± 0.7 mV. R_{te} was significantly increased to 42.1 ± 5.8 Ωcm^2 compared to non-CF ($n=10$). In the rectal mucosa of non-CF subjects, perfusion with the cyclo-oxygenase inhibitor indomethacin (10 μmol l^{-1}; basolateral side) largely attenuated CFTR activity and reduced lumen negative I_{sc} ($\Delta I_{sc}=21.9\pm3.7$ μA cm^{-2}; $n=12$), while the opposite was observed in tissues from CF patients ($\Delta I_{sc}=-6.4\pm1.7$ μA cm^{-2}; $n=10$). This increase in lumen negative I_{sc} is caused by deactivation of luminal cyclic AMP activated K^+ channels as described in a previous report (Mall *et al*., 2000). Amiloride-sensitive Na^+ conductance was significantly increased in rectal biopsies from CF patients ($\Delta I_{sc}=32.9\pm10.6$ μA cm^{-2}; $n=10$) compared to non-CF individuals ($\Delta I_{sc}=9.9\pm2.5$ μA cm^{-2}; $n=12$). Stimulation of the tissue with IBMX/forskolin in the presence of amiloride

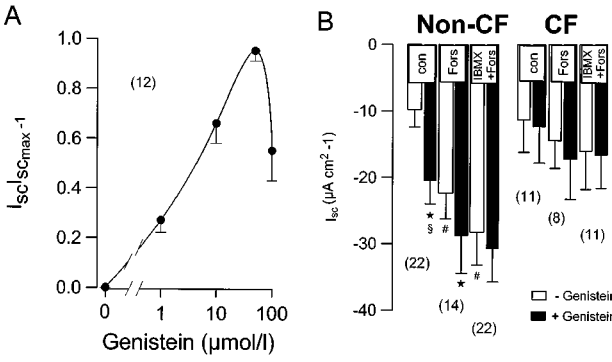


Figure 2 Effect of genistein on ion transport in nasal epithelia from non-CF and CF subjects. (A) Concentration response curve for the effect of genistein (under resting conditions) in non-CF respiratory epithelia, showing an EC_{50} of about 3.8 μmol l^{-1}. An inhibitory effect was observed at concentrations higher than 50 μmol l^{-1}. (B) Summary of the effects of 50 μmol l^{-1} genistein (mucosal side) on non-CF and CF nasal epithelia. Experiments were performed in the presence of amiloride (10 μmol l^{-1}; mucosal side) and in either (i) absence of cyclic AMP dependent stimulation, (ii) after submaximal stimulation with low concentrations of forskolin (0.1 μmol l^{-1}; basolateral side) or (iii) after maximal stimulation with IBMX and forskolin (100 μmol l^{-1} and 1 μmol l^{-1}; both sides). #Significant effects of forskolin and IBMX/forskolin compared to control (paired *t*-test). *Indicates statistical significance for the effect genistein compared to control and in the presence of forskolin (paired *t*-test). §Indicates statistical significance when comparing the effect of genistein under the different experimental conditions (see above) in non-CF and CF tissues (unpaired *t*-test). (Number of subjects).

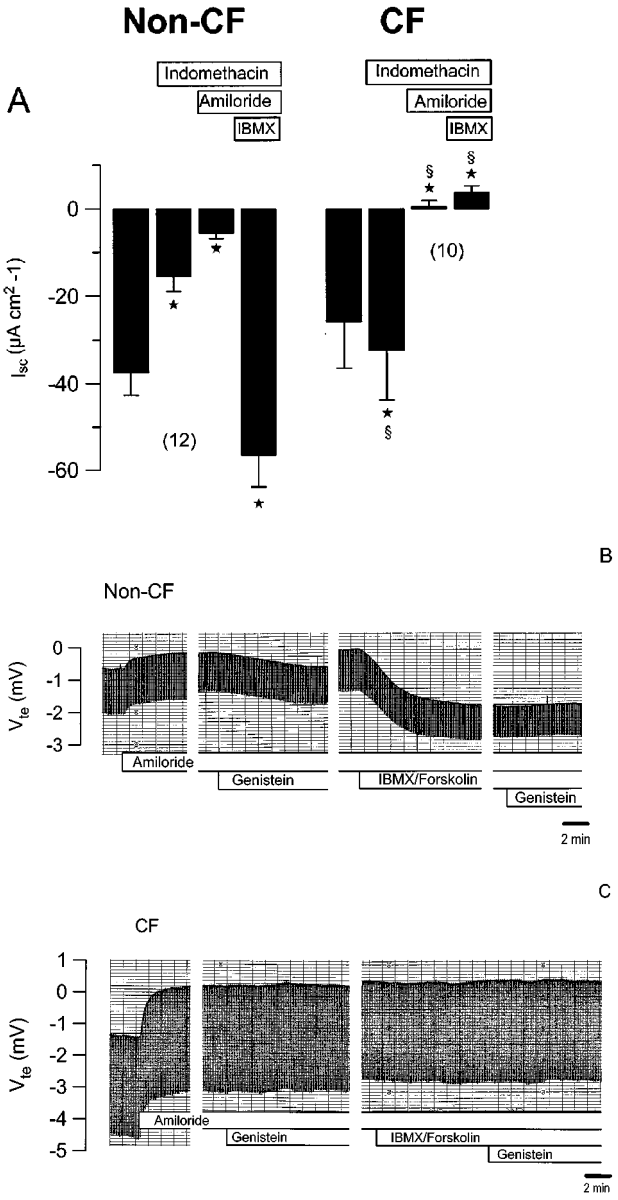
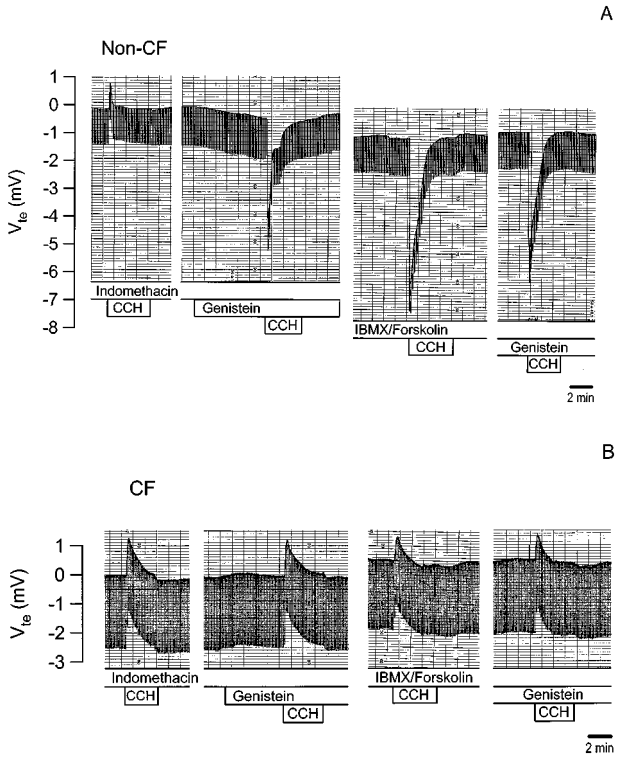


Figure 3 (A) Summary of short circuit currents (I_{sc}) in rectal epithelia from non-CF and CF individuals under resting conditions, after incubation with indomethacin (10 μmol l^{-1}; basolateral side), after blocking epithelial Na^+ channels by amiloride (10 μmol l^{-1}; mucosal side) and after activation of ion transport by IBMX and forskolin (100 μmol l^{-1} and 1 μmol l^{-1}; basolateral side). *Indicates statistical significance for the effects of indomethacin, amiloride and IBMX/forskolin in non-CF or CF (paired *t*-test). §Indicates statistical significance when comparing the effects of indomethacin, amiloride and IBMX/forskolin obtained in experiments from rectal tissues of non-CF and CF subjects (unpaired *t*-test). (Number of subjects). Effect of genistein (50 μmol l^{-1}; mucosal side) on transepithelial voltage (V_{te}) in (B) non-CF and (C) CF human rectal mucosa. The effects of genistein were examined in both absence and presence of IBMX and forskolin and experiments were carried out in the presence of indomethacin and amiloride. R_{te} was continuously determined from the V_{te} downward deflections obtained by pulsed current injection.

activated a lumen negative Cl^- secretory response in non-CF ($\Delta I_{sc} = -47.1 \pm 6.0$ μA cm^{-2}; $n = 12$), while the opposite was observed in tissues from CF patients ($\Delta I_{sc} = 3.3 \pm 1.4$ μA cm^{-2}; $n = 10$). This lumen positive I_{sc} in CF has recently been identified as a luminal cyclic AMP-dependent K^+ conductance (Mall *et al*., 1998b). These results resemble typical features of non-CF and CF rectal epithelia which have been described originally (Hardcastle *et al*., 1991; Veeze *et al*., 1991) and which were confirmed in previous reports (Mall *et al*., 1998b; 2000) (Figure 3A). Next the effects of genistein (50 μmol l^{-1}; mucosal side) were assessed in paired experiments (i) after deactivation of CFTR by indomethacin, (ii) after submaximal (0.1 μmol l^{-1} forskolin, basolateral side) and (iii) maximal (100 μmol l^{-1} IBMX and 1 μmol l^{-1} forskolin; both sides) activation of CFTR. Following incubation with indomethacin, genistein slightly but significantly increased Cl^- secretion and I_{sc} in rectal epithelium from non-CF individuals (Figures 3B and 4B). The EC_{50} for the effects of genistein was of 35 μmol l^{-1} (Figure 4A). In this series of experiments maximum genistein responses in rectal biopsies were observed at a concentration of 100 μmol l^{-1} with an absolute magnitude of -14.9 ± 3.5 μA cm^{-2} ($n = 7$). Stimulation of I_{sc} by genistein (50 μmol l^{-1}) was even enhanced after submaximal activation of CFTR but the opposite, namely inhibition of I_{sc} was detected after maximal activation of cyclic AMP dependent pathway. In CF rectal tissues genistein failed to activate any I_{sc} response independent of cyclic AMP stimulation (Figures 3C and 4B).

It has been shown that cholinergic stimulation enhances CFTR-mediated Cl^- secretion by activating basolateral Ca^{2+}-dependent K^+ channels and thus increasing the driving force for Cl^- secretion (Mall *et al*., 1998b). To further investigate the effects of genistein on luminal CFTR Cl^- conductance and basolateral Ca^{2+}-dependent K^+-conductance, the Ca^{2+}-mobilizing agonist carbachol (CCH, 100 μmol l^{-1}; basolateral side) was added in the absence or presence of genistein. When CFTR activity was lost in the presence of indomethacin, CCH induced a lumen positive response in non-CF rectal tissues which was caused by activation of luminal K^+ channels as



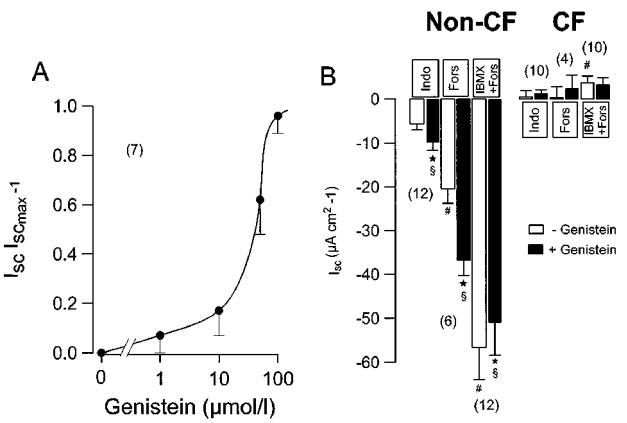


Figure 4 Effect of genistein on ion transport in rectal tissues from non-CF and CF individuals. (A) Concentration response curve for the effect of genistein (in the presence of 10 μmol l^{-1} indomethacin; basolateral side) on non-CF rectal mucosa ($EC_{50} = 35$ μmol l^{-1}). (B) Summary of the effects of 50 μmol l^{-1} genistein (mucosal side) on non-CF and CF rectal mucosa. Experiments were performed in the presence of amiloride (10 μmol l^{-1}; mucosal side) and in either(i) absence of cyclic AMP dependent stimulation, (ii) after submaximal stimulation with low concentrations of forskolin (0.1 μmol l^{-1}; basolateral side) or (iii) after maximal stimulation with IBMX and forskolin (100 μmol l^{-1} and 1 μmol l^{-1}; basolateral side). # Indicates statistical significance for the effects of forskolin and IBMX/forskolin compared to control (paired *t*-test). *Indicates statistical significance for the effect of genistein in the absence of cyclic AMP activation, in the presence of forskolin and in the presence of IBMX/forskolin (paired *t*-test). §Indicates statistical significance when comparing the effect of genistein under the different experimental conditions (see above) in non-CF and CF tissues (unpaired *t*-test). (Number of subjects).

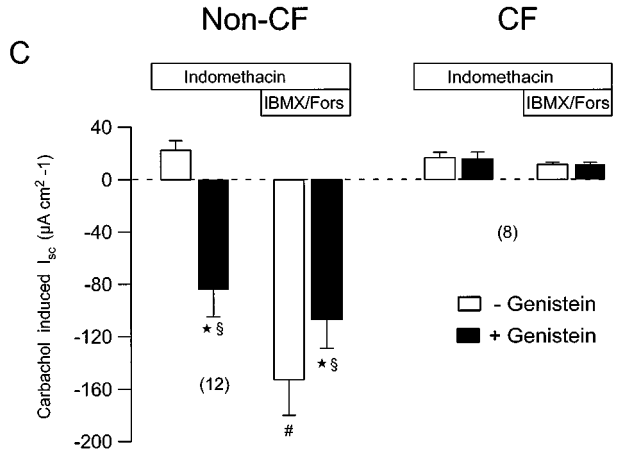


Figure 5 Effects of genistein on cholinergic secretion after deactivating (indomethacin) or activating (forskolin/IBMX) CFTR. Original recordings of V_{te} obtained from rectal biopsies from (A) non-CF and (B) CF subjects. All experiments were performed in the presence of indomethacin (10 μmol l^{-1}; basolateral side) and amiloride (10 μmol l^{-1}; mucosal side). Cholinergic stimulation was induced by carbachol (CCH, 100 μmol l^{-1}; basolateral side). (C) Summary of the CCH induced I_{sc} in CF and non-CF rectal biopsies in the absence and presence of genistein (50 μmol l^{-1} mucosal side) and dependence on stimulation with IBMX and forskolin (100 μmol l^{-1} and 1 μmol l^{-1}; basolateral side). #Indicates significant difference for CCH induced I_{sc} in the presence of IBMX/forskolin compared to indomethacin (paired *t*-test). *Indicates statistical significance for the effect of genistein on CCH induced I_{sc} in the presence of indomethacin and IBMX/forskolin (paired *t*-test). §Indicates statistical significance when comparing the effects of genistein on CCH induced I_{sc} in experiments obtained from non-CF and CF tissues (unpaired *t*-test). (Number of subjects).

shown previously (Mall *et al*., 1998b) (Figure 5). In the presence of both indomethacin and genistein the cholinergic response was reversed and CCH induced a large lumen negative response. These data show that genistein is able to activate CFTR in the absence of cyclic AMP dependent stimulation. When CCH was applied after stimulation of CFTR (IBMX/forskolin) large lumen negative V_{te} and I_{sc} were activated which were reduced when genistein was applied simultaneously. This result suggests an inhibitory effect of genistein on basolateral Ca^{2+}-dependent K^+-channels as reported previously for CFTR expressing cell lines and rat distal colon (Diener & Hug, 1996; Illek *et al*., 1996). In CF, due to the defect of luminal CFTR Cl^- channels, genistein had no effect on the lumen positive CCH response independent of absence or presence of IBMX/forskolin (Figure 5B,C).

Effects of genistein on ion channels expressed in Xenopus *oocytes*

The results obtained in the native tissue suggest that genistein might have multiple effects on ion conductances, such as luminal CFTR Cl^- channels as well as basolateral K^+ channels. We therefore examined the effects of genistein on CFTR when expressed in *Xenopus* oocytes. As shown in Figure 6A, genistein dose dependently activated a whole cell Cl^- conductance in both presence and absence of cyclic AMP dependent stimulation. This was observed for wtCFTR as well as ΔF508-CFTR. However, the effects on ΔF508-CFTR were relatively small compared to those obtained for wtCFTR. In our hands, pre-stimulation with IBMX (1000 μmol l^{-1}) and forskolin (2 μmol l^{-1}) only slightly enhanced genistein activated Cl^- conductance in both wtCFTR and ΔF508-CFTR (Figure 6). EC_{50} values for the effect of genistein were approximately 9 μmol l^{-1} (wtCFTR) and 5 μmol l^{-1} (ΔF508-CFTR) in the absence of IBMX/forskolin and were 6 μmol l^{-1} (wtCFTR) and 1.5 μmol l^{-1} (ΔF508-CFTR), respectively, after stimulation with IBMX/forskolin. Because the data on the intact tissue suggest inhibition of basolateral K^+ channels, we further examined the effects of genistein on two recently cloned epithelial K^+ channels: K_vLQT1- and SK4. K_vLQT1 is most likely forming part of the basolateral cyclic AMP activated K^+ channel in both colon and airways (Mall *et al*., 1998b). SK4 (IK1) was cloned recently and was demonstrated to form the basolateral Ca^{2+} activated K^+ channel in colonic crypt cells (Warth *et al*., 1999; Joiner *et al*., 1997). As shown in Figure 7, lower concentrations of genistein had no effect on rK_vLQT1 currents expressed in *Xenopus* oocytes, while about 20% of the K^+ current were inhibited at a concentration of 50 μmol l^{-1} (Figure 7). hSK4 was coexpressed with the $P2Y_2$ receptor to incorporate a receptor coupled to the Ca^{2+}

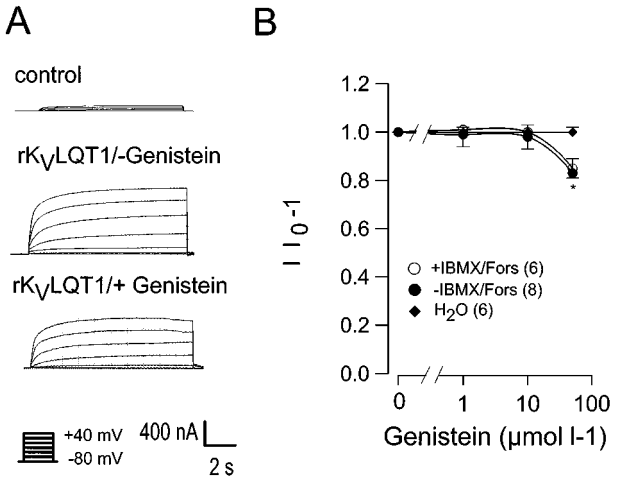


Figure 7 Effect of genistein on the cyclic AMP regulated rK_vLQT1 K^+ channel expressed in *Xenopus* oocytes. (A) Delayed voltage activated whole cell K^+ currents generated by rK_vLQT1 according to the voltage pulse protocol shown. Genistein (50 μmol l^{-1}) slightly inhibited voltage activated rK_vLQT1 K^+ currents. (B) Concentration response curve for the effect of genistein on rK_vLQT1 in both absence and presence of IBMX (1000 μmol l^{-1}) and forskolin (2 μmol l^{-1}). Genistein significantly inhibited rK_vLQT1 K^+ currents at higher concentrations (50 μmol l^{-1}). *Indicates statistical significance for the effect of genistein on rK_vLQT1 mediated K^+ currents (paired *t*-test). (Number of experiments).

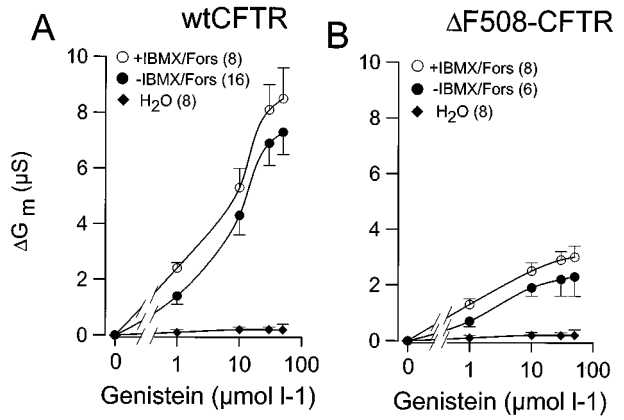


Figure 6 Concentration response curves for the effects of genistein on whole cell Cl^- conductances (ΔG_m) measured in *Xenopus* oocytes expressing (A) wtCFTR or (B) ΔF508-CFTR and water injected control oocytes. Effects of genistein were examined in both presence or absence of cyclic AMP dependent stimulation. EC_{50} values for the effect of genistein were 9 μmol l^{-1} (wtCFTR) and 2.5 μmol l^{-1} (ΔF508-CFTR) in the absence of IBMX/forskolin and were 7.4 μmol l^{-1} (wtCFTR) and 1.5 μmol l^{-1} (ΔF508-CFTR), respectively, after stimulation with IBMX (1000 μmol l^{-1}) and forskolin (2 μmol l^{-1}).

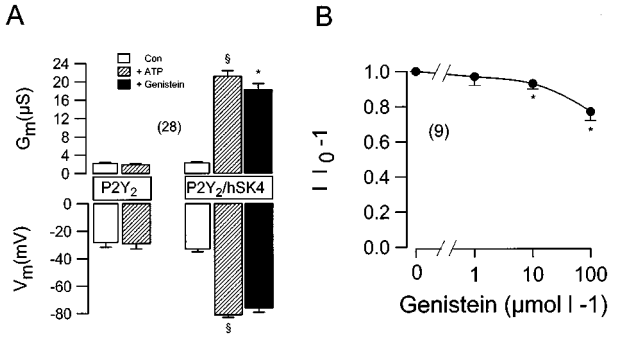


Figure 8 Effects of genistein on Ca^{2+} activated hSK4 K^+ channels expressed in *Xenopus* oocytes. The purinergic receptor type $P2Y_2$ was coexpressed with the Ca^{2+} sensitive K^+ channel hSK4 or was expressed in the absence of hSK4. Cl^- was replaced in the extracellular bath solution by gluconate except of 5 mmol l^{-1}. (A) Summary of the effects of 100 μmol l^{-1} ATP on oocytes expressing either $P2Y_2$ or coexpressing $P2Y_2$/hSK4. Genistein (10 μmol l^{-1}) slightly but significantly inhibited ATP induced K^+ conductance in $P2Y_2$/hSK4 coexpressing oocytes. (B) Concentration response curve for the effects of genistein on $P2Y_2$/hSK4 coexpressing oocytes. Genistein inhibited rhSK4 K^+ currents at concentrations of 10 μmol l^{-1} and higher. §Indicates statistical significance for the effect of ATP on rhSK4 mediated K^+ currents (paired *t*-test). *Indicates statistical significance for the effect of genistein on rhSK4 K^+ currents (paired *t*-test). (Number of experiments).

signalling pathway into *Xenopus* oocytes. When extracellular Cl^- was replaced by gluconate, increase of intracellular Ca^{2+} by adding ATP (100 μmol l^{-1}), did not activate any current in oocytes expressing $P2Y_2$ receptors alone (Figure 8A). However, in oocytes coexpressing $P2Y_2$ and hSK4, a large potassium conductance was activated by ATP and the membrane voltage was hyperpolarized (Figure 8B). The ATP activated whole cell conductance was inhibited slightly but significantly by genistein. Figure 8B shows that a genistein concentration of 10 μmol l^{-1} and high does significantly inhibit the Ca^{2+} activated K^+ conductance in *Xenopus* oocytes. Because similar inhibition was-observed when hSK4 was activated by ionomycin (data not shown), we conclude that genistein directly inhibits Ca^{2+} activated K^+ channels at higher concentrations.

Discussion

In this paper we have performed a detailed analysis of the effects of genistein in native human respiratory and rectal epithelia and on ion channels contributing to epithelial ion secretion, such as CFTR and putative basolateral cyclic AMP and Ca^{2+} activated K^+ channels (Mall *et al*., 1998b; Murthy & Makhlouf, 1998). To be able to study the effect of genistein in very small native human tissue samples we made use of a micro-Ussing chamber with an exposed tissue area of only 0.95 mm^2. Due to edge leak conductance the absolute magnitude of measured V_{te} and R_{te} were certainly underestimated compared to *in vivo* conditions. Despite imperfect edge sealing we observed robust responses to amiloride and cyclic AMP stimulation. Furthermore CF typical alterations of ion transport (e.g. increased Na^+ absorption and a defect in cyclic AMP-dependent Cl^- secretion) were well preserved in native human nasal and rectal tissues. The lack of Cl^- conductance that is caused by mutations in the CFTR Cl^- channel prompted research on putative activators of CFTR that could be helpful in the treatment of cystic fibrosis. Illek *et al*. reported stimulatory effects of genistein and several other flavonoids on CFTR Cl^- conductance in airway epithelial cells in culture and stimulation of Cl^- secretion *in vivo* by measuring nasal potential difference in non-CF volunteers (Illek *et al*., 1996; Illek & Fischer, 1998). Initial observations prompted the suggestion that genistein blocks phosphatases which normally inhibit CFTR activity (Illek *et al*., 1996; Reenstra *et al*., 1996). Meanwhile several mechanisms have been proposed for the effects of genistein on CFTR, including facilitation of PKA-mediated phosphorylation or inhibition of dephosphorylation leading to an increase in the channel open probability (Shuba & McDonald, 1997; Chiang *et al*., 1997; Weinreich *et al*., 1997). However, subsequent studies showed that genistein acts in a tyrosine kinase and phosphatase–independent way by interacting with the second nucleotide binding domain of CFTR (French *et al*., 1997). Another study showed that in fact ATPase and GTPase activity of the second nucleotide binding fold is inhibited by genistein (Randak *et al*., 1999). It has been reported that genistein is able to potentiate Cl^- channel activity of wtCFTR and ΔF508-CFTR after pre-stimulation with submaximal concentrations of forskolin (Hwang *et al*., 1997). Effects of genistein were observed in airway cells as well as colonic epithelial cells and over-expressing cells (Sears *et al*., 1995; Illek & Fischer, 1998; Illek *et al*., 1995). In *Xenopus* oocytes, direct action of genistein on CFTR was demonstrated which was potentiated by forskolin and IBMX (Weinreich *et al*., 1997).

A low cyclic AMP dependent activation was required in most studies in order to facilitate activation of CFTR by genistein (Shuba & McDonald, 1997; Hwang *et al*., 1997; Diener & Hug, 1996). In the present experiments with *Xenopus* oocytes we found activation of wtCFTR by genistein even in the absence of cyclic AMP enhancing agonists. This result was confirmed by activation of Cl^- secretion in human non-CF respiratory and rectal epithelia in the absence of parallel stimulation with IBMX/forskolin. Moreover, genistein enabled CCH mediated Cl^- secretion in rectal mucosa which was incubated with indomethacin. Indomethacin inhibits cyclooxygenase mediated generation of cyclic AMP and thus inactivates CFTR (Mall *et al*., 1998b). Because CFTR is probably the only luminal Cl^- conductance in the human rectal epithelium, an increase of intracellular Ca^{2+} by CCH results in K^+ secretion under these conditions. Genistein reversed the CCH response and enabled CCH mediated Cl^- secretion, a clear indication that genistein is able to activate wtCFTR in the absence of any pre-stimulation by cyclic AMP. Moreover, potentiation of the genistein effect by submaximal stimulation with forskolin was only observed in the non-CF rectal mucosa but not in the nasal tissue. In *Xenopus* oocytes overexpressing mutant ΔF508-CFTR, genistein either in absence or presence of parallel stimulation with IBMX/forskolin activated a Cl^- conductance which was in the range of 20% compared to wtCFTR.

In the present study we examined the effect of genistein on tissues derived from CF patients carrying ΔF508 CFTR on at least one allele and some patients were shown to be compound heterozygous with a second severe mutation (R553X, N1303K, 3905insT, G551D). It is shown that genistein failed to activate Cl^- secretion in either nasal or rectal mucosa of these CF genotypes, independent of pre-stimulation with low concentration of forskolin or using a cocktail of IBMX and forskolin. These and previous data demonstrate that genistein is able to activate some Cl^- conductance generated by mutant CFTR overexpressed in cell lines or in *Xenopus* oocytes. In native CF tissues the effect of genistein was negligible and thus no Cl^- secretion was observed. However, our data do not exclude that genistein may have some effect in CF patients carrying mild CFTR mutations that are inserted into the plasma membrane but which cause reduced Cl^- channel function or quantity or in conjunction with other drugs that increase trafficking of mutant CFTR to the plasma membrane.

However, apart from the limited effects of genistein in the native epithelium, genistein had additional inhibitory effects on basolateral K^+ conductance in both respiratory and rectal epithelium when applied at higher concentrations. In a previous report, the most abundant flavonoid quercetin has been demonstrated to activate CFTR Cl^- conductance but was also shown to inhibit carbachol induced Cl^- secretion in rat colon (Cermak *et al*., 1998). Diener & Hug (1996) have shown stimulation of I_{sc} by genistein in rat colon, but also reported inhibition of basal K^+ conductance and Ca^{2+} mediated ion secretion. Inhibition of Ca^{2+} mediated secretion in rat colon was caused by inhibition of basal and Ca^{2+} activated basolateral K^+ channels. In that respect it is important to recall another study showing inhibition of carbachol stimulated Ca^{2+} entry in HT_{29} colonic carcinoma cells (Bischof *et al*., 1995). Finally, inhibitory effects of genistein on Cl^- conductance been observed in cardiac myocytes (Obayashi *et al*., 1999). We also detected inhibition of epithelial ion transport, particularly at higher concentrations of genistein. The data indicate that probably both cyclic AMP dependent as well as Ca^{2+} activated K^+ conductance are inhibited by high concentrations of genistein. Both

basolateral K^+ conductances have been shown to increase the driving force for Cl^- secretion in epithelia and both have been identified on the molecular level in the meantime: K_vLQT1 is the essential component of the cyclic AMP activated K^+ channel (Mall *et al*., 1998b; Warth *et al*., 1996). SK4 is very likely to form Ca^{2+} activated K^+ channels (Warth *et al*., 1999; Joiner *et al*., 1997). It has been demonstrated recently that, depending on the expression system, K^+ channels may have quite different properties and therefore, results which have been obtained during expression of the channel in *Xenopus* oocytes may not necessarily be applicable to the native tissue (Shah & Haylett, 2000). However, we expressed both types of K^+ channels in *Xenopus* oocytes and confirmed the inhibitory effects of higher concentrations of genistein on both rK_vLQT1 and hSK4. While activation of CFTR is promoting ion secretion in airways and rectum, inhibition of both K^+ channels would limit this stimulatory effect and eventually even reduce ion secretion. Taken together these results and the fact that genistein was without any stimulatory effects in human CF airways and rectal biopsies, do not support the use of genistein in the treatment of cystic fibrosis. Furthermore non-specific inhibition of phosphatases and tyrosine kinases by genistein could induce numerous side effects. These problems might be overcome by further studies on related compounds which act more specifically on CFTR (Kunzelmann, 1999; Schultz *et al*., 1999).

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