Genistein activates CFTR-mediated Cl\(^{-}\) secretion in the murine trachea and colon

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Goddard, Catharine A., Martin J. Evans, and William H. Colledge. Genistein activates CFTR-mediated Cl\(^{-}\) secretion in the murine trachea and colon. Am J Physiol Cell Physiol 279: C383–C392, 2000.—The action of the isoflavone genistein on the cystic fibrosis transmembrane conductance regulator (CFTR) has been studied in many cell systems but not in intact murine tissues. We have investigated the action of genistein on murine tissues from normal and cystic fibrosis (CF) mice. Genistein increased the short-circuit current (I\(_{sc}\)) in tracheal (16.4 ± 2.8 \(\mu\)A/cm\(^2\)) and colonic (40.0 ± 4.4 \(\mu\)A/cm\(^2\)) epithelia of wild-type mice. This increase was inhibited by furosemide, diphenylamine-2-carboxylate, and glibenclamide, but not by DIDS. In contrast, genistein produced no significant change in the I\(_{sc}\) of the tracheal epithelium (0.9 ± 1.1 \(\mu\)A/cm\(^2\)) and decreased the I\(_{sc}\) of colons from CF null (−13.1 ± 2.3 \(\mu\)A/cm\(^2\)) and ΔF508 mice (−10.3 ± 1.3 \(\mu\)A/cm\(^2\)). Delivery of a human CFTR cDNA-liposome complex to the airways of CF null mice restored the genistein response in the tracheae to wild-type levels. Tracheas from ΔF508 mice were also studied: 46% of trachea showed no response to genistein, whereas 54% gave an increase in I\(_{sc}\) similar to that in wild type. We conclude that genistein activates CFTR-mediated Cl\(^{-}\) secretion in the murine trachea and distal colon.

Cystic fibrosis transmembrane conductance regulator; short-circuit current; chloride channel blockers; ΔF508

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Genistein is a naturally occurring isoflavone that was initially described as a tyrosine kinase inhibitor (1) and has subsequently been found to inhibit topoisomerase II (24), histone kinase (13), and \(P_{1}\)-purinergic receptors (27). Genistein has also been shown to regulate a number of ion transporters including the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (26, 39), voltage-gated K\(^{+}\) channels in vascular smooth muscle (36), the Ca\(^{2+}\)-dependent K\(^{+}\) conductance in rat distal colon (6), a K\(^{+}\) channel in the basolateral membrane of epithelial cells (16), the Na\(^{+}\)/H\(^{+}\) exchanger (7), and the multidrug resistance-associated protein 1 (38). Illeck et al. (18) were the first to show that in contrast to its inhibitory actions, genistein at low concentrations (≤50 \(\mu\)M) could activate the cystic fibrosis transmembrane conductance regulator (CFTR) expressed in NIH/3T3 fibroblasts. Further studies have shown that genistein stimulates CFTR-mediated Cl\(^{-}\) secretion in the shark rectal gland (21), in cell lines derived from human colonic epithelia (15, 16, 32), and in Hi-5 insect cells (40). These reports all concluded that genistein increases CFTR-mediated Cl\(^{-}\) secretion without increasing the intracellular concentration of either cAMP ([cAMP]\(_{i}\)) or Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)). Initial studies by Lehrich and Forrest (21) and Illeck et al. (18) both suggested that genistein activates CFTR through inhibition of tyrosine kinase pathways, acting either directly on CFTR or on regulators of CFTR. Further work by Reenstra et al. (31), Illeck et al. (16), and Yang et al. (42) showed that a basal level of protein kinase A (PKA) activity is required for activation of CFTR by genistein. They concluded that genistein acts by inhibiting phosphatases. Reenstra et al. (31) showed that the level of CFTR phosphorylation increased under stimulation by genistein. Illeck et al. (16) demonstrated that whereas genistein does not increase [cAMP]\(_{i}\), it does require cellular cAMP to regulate CFTR. More recent experiments have provided evidence that genistein binds directly to CFTR, most probably in one or both of the nucleotide binding domains, although binding to another domain cannot be excluded (8, 15, 40, 41). It has been demonstrated that genistein binds to the nucleotide binding folds of the tyrosine kinase HCK (35) and topoisomerase II (24). The current data suggest that CFTR activation by genistein involves binding of genistein to CFTR and prior phosphorylation of the regulatory domain by PKA (8, 15, 40). The variability in genistein-mediated Cl\(^{-}\) secretion in different cell systems and its differing reliance on rising intracellular cAMP levels could be explained by different basal levels of PKA activity giving different basal levels of CFTR phosphorylation. In some cases this level of phosphorylation may not be sufficient to allow CFTR activation by genistein without additional phosphorylation by cAMP-stimulated PKA activity.

Recently, Illeck and Fischer (15) have shown that genistein and other naturally occurring flavonoids activate CFTR-mediated Cl\(^{-}\) secretion in Calu-3 monolayers and stimulate in vivo nasal potential difference by almost 28% of the isoproterenol response. Hwang et

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al. (14) have shown that genistein is able to prolong the open time of ΔF508-CFTR channels expressed in NIH/3T3 cells. These channels normally show a prolonged closed time compared with maximally activated wild-type channels, but in the presence of genistein and forskolin the open probabilities of ΔF508-CFTR and wild-type channels are the same (14). It has recently been demonstrated that genistein can also activate the G551D mutant CFTR channel in HeLa cells and in cystic fibrosis (CF) patients (19). These findings suggest that the regulation of CFTR by genistein and/or other flavonoids may be of therapeutic value in the treatment of CF. Mouse models provide a suitable testing ground for novel gene therapy, and pharmacological strategies to treat CF as electrophysiological techniques can be used to detect the presence of functional CFTR in several relevant tissues. Therefore, we have investigated the effect of genistein on CFTR-mediated Cl⁻ secretion in the murine trachea and colon. Short-circuit current (Isc) responses to genistein and to other Cl⁻ channel activators and inhibitors were compared in wild-type, CF null (Cftr<sup>tm1Cam</sup>) (30), and ΔF508 (Cftr<sup>tm2Cam</sup>) (4) mice. The results show that genistein is able to increase Cl⁻ secretion in the trachea and colon and that the increase is dependent on the presence of the CFTR channel.

**MATERIALS AND METHODS**

**Animals.** Tissues were taken from Cambridge CF null Cftr<sup>tm1Cam</sup> or ΔF508 Cftr<sup>tm2Cam</sup> mouse stocks. Both lines are maintained as outbred stocks (129Sv/Ev × MF1). Wild-type tissues were taken from either +/+ or +/- mice and treated as one group because no statistical difference was found between these genotypes. More wild-type mice than CF or ΔF508 mice were used in this study; therefore, this group consists of littermates of CF and ΔF508 mice used in the study and age-matched mice from other litters. Because the stocks are outbred, mice from many different matings were studied to ensure that results were representative of the electrophysiological status of the whole stock. CF tissues were taken from mice homozygous for either the CF null mutation or the ΔF508 mutation. The mice were between 40 and 105 days old, except for four mice used for null tracheal measurements, which were between 330 and 356 days old. The tracheas of these four mice showed no differences in their response to drugs compared with the response of CF null mice 41–62 days old.

**Measurement of electrogenic transepithelial ion movement by Isc.** Mice were killed by exposure to a rising concentration of CO<sub>2</sub>. The trachea was removed and stripped of connective tissue and muscle, opened longitudinally, and mounted in an Ussing chamber so that a surface area of 2.3 mm<sup>2</sup> was exposed. The muscle layer was stripped from the distal colonic epithelium, which was mounted in an Ussing chamber so that a surface area of 20 mm<sup>2</sup> was exposed. A parafilm washer was placed between the two halves of the Ussing Chamber to reduce edge damage. The tissue was bathed on one side by 20 ml of Krebs-Henseleit solution (KHS) maintained at 37°C and gassed with 95% O<sub>2</sub>–5% CO<sub>2</sub>. Tissues were short-circuited using a WPI DVC-1000 voltage clamp (World Precision Instruments, Stevenage, UK). Changes in voltage were monitored by calomel electrodes placed in a reservoir of 3 M KCl and linked to the Ussing chamber by KHS-filled polythene tubes, the ends of which were plugged with 3 M KCl-1.5% agarose. Current was passed from the WPI clamp using Ag-AgCl electrodes via 3 M KCl agar bridges. The Isc was recorded on a MacLab (ADInstruments, Hastings, UK) using the Chart acquisition program run on an Apple PowerPC. Drugs were added to either the apical or basolateral bathing solution, or both. KHS had the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25.0 NaHCO<sub>3</sub>, and 11.1 glucose. The solution had a pH of 7.4 when maintained at 37°C and bubbled with 95% O<sub>2</sub>–5% CO<sub>2</sub>.

**Drugs.** Amiloride (Sigma) was made as a 10 mM stock solution in water and was used at a final concentration of 100 μM. Genistein (synthetic, Sigma) was made as a 100 mM stock solution in DMSO and was used at a final concentration of 50 or 100 μM. A-23187 (Sigma) was kept as a 1 mM stock solution in 95% ethanol and was used at 1 μM. 2,5-Di-tert-butyl-hydroquinone (TBHQ; Aldrich) was made as a 25 mM stock solution in 95% ethanol and was used at 25 μM. Forskolin (Calbiochem) was kept as a 10 mM stock solution in 95% ethanol and was used at a final concentration of 10 μM. Furosemide (Sigma) was dissolved in water with a drop of NaOH (~100 μl of NaOH in 5 ml of water) to form a stock solution of 100 mM and was used at a final concentration of 1 mM. Diphenylamine-2-carboxylate (DPC; Aldrich) was stored as a 100 mM stock solution in 95% ethanol and was used at a final concentration of 1 mM. 4,4’-Diisothiocyanato-stilbene-2,2’-disulfonic acid (DIDS; Sigma) was made as a 300 mM stock solution in DMSO and was used at a final concentration of 300 μM. Glibenclamide (Sigma) was dissolved in DMSO and kept as 1 and 100 mM stock solutions that were used at a final concentration of 1 or 100 μM, respectively. Drugs were added sequentially at 10-min intervals, except for A-23187 and TBHQ, which were added together and left for 20 min before further drugs were added.

**CFTR gene delivery to CF null mice.** CF null mice were anesthetized with Avertin (0.5 g of 2,2,2-tribromoethanol dissolved in 0.63 ml of tertiary amyl alcohol and then diluted 1:50 in PBS) at a dose of 0.017 ml/g body weight. The 100 μl of DNA-liposome mix was instilled into the trachea over a 10-min period. The DNA-liposome mix consisted of 10 μg of DC-Chol:DOPE [3β-[N-(N’,N’-dimethylaminoethane)carbomyl] cholesterol:dioloyl-phosphatidyl ethanolamine] (9) in a Krebs-HEPES pH 9.0 diluent. The charge ratio of the complex is 1.98. Mice received one of two plasmids. pTRIAL10CFTR2 (pT10CFTR) contains the human CFTR (hCFTR) cDNA under the control of the Rous Sarcoma Virus 3’ LTR promoter (22). pTRAIL10 (pT10) is the plasmid backbone lacking the hCFTR cDNA.

**Statistics.** Results are presented as means ± SE. Comparisons of results were made using an unpaired, two-tailed Student’s t-test. P = 0.05 was considered significant.

**RESULTS**

Genistein increases Cl⁻ secretion in wild-type tracheas. The effect of genistein on Cl⁻ secretion in wild-type tracheas was examined (Fig. 1). Amiloride (100 μM) was always added to the apical membrane before genistein to block Na⁺ absorption by the epithelial Na⁺ channel (ENaC) because electrogenic Na⁺ absorption reduces the electrochemical gradient for Cl⁻ secretion. Amiloride addition caused a reduction in baseline Isc by 16.4 ± 2.8 μA/cm² (n = 15) (Fig. 1A). Addition of 50 μM genistein increased Isc by 16.4 ± 2.8 μA/cm² (n = 15) (Fig. 1A).
apical membrane increased $I_{sc}$ by $22.8 \pm 3.6 \mu A/cm^2$ ($n = 6$), which was not significantly different from the increase in $I_{sc}$ when genistein was added to both membranes. Genistin (100 $\mu$M), an inactive form of genistein (1), produced no change in $I_{sc}$ when added bilaterally ($n = 4$, data not shown).

The response to 50 $\mu$M genistein could not be augmented by increasing the phosphorylation level of CFTR before genistein addition. The addition of either 100 nM forskolin or 1 $\mu$M IBMX, after amiloride, produced $I_{sc}$ increases of $12.9 \pm 3.1$ ($n = 5$) and $12.3 \pm 3.7$ $\mu A/cm^2$ ($n = 7$), respectively. Genistein produced a similar further increase in $I_{sc}$ after both drugs, $14.6 \pm 3.6$ $\mu A/cm^2$ after forskolin ($n = 5$) and $15.2 \pm 5.0$ $\mu A/cm^2$ after IBMX ($n = 7$). These responses are not significantly different from the response to genistein without prior addition of low concentrations of forskolin or IBMX.

The effect of $Cl^-$ channel blockers on the genistein response was studied. DPC (1 $\mu$M) (2) inhibited all of the genistein-induced $I_{sc}$ increase and part of the basal $I_{sc}$, suggesting that a proportion of the basal $I_{sc}$ was due to $Cl^-$ secretion (Fig. 1A). Apical addition of 300 $\mu$M DIDS (2) had no significant effect on genistein-stimulated $I_{sc}$ (mean change $-2.3 \pm 2.7$ $\mu A/cm^2$, $n = 6$) (Fig. 1B). In contrast, apical addition of 100 $\mu$M glibenclamide (33) inhibited the genistein-sensitive increase in $I_{sc}$ by 84.7 $\pm 24.2\%$ ($n = 7$) (Fig. 1B), whereas addition of 1 $\mu$M glibenclamide to the apical membrane had no effect on the genistein response ($n = 6$, data not shown). After genistein was added bilaterally or to the apical membrane only, the Na$^+-K^+$-$2Cl^-$ cotransporter inhibitor furosemide (1 mM) inhibited the genistein response by $79.3 \pm 14.9\%$ ($n = 12$) (Fig. 1, C and D). In $Cl^-$-free KHS the response to genistein was reduced by $\sim70$ to $3.9 \pm 2.0 \mu A/cm^2$ ($n = 8$), significantly smaller than the response in normal KHS ($P < 0.01$) (Fig. 1D).

The effect of genistein on tracheas taken from CF null mice ($Cftr^{TM1Cam}$) was investigated (Fig. 2). The response to amiloride (100 $\mu$M) was similar in wild-type ($-34.9 \pm 7.1 \mu A/cm^2$, $n = 15$) and CF null tracheas ($-26.6 \pm 5.2 \mu A/cm^2$, $n = 9$) (Fig. 2, A and B). Bilateral addition of 50 $\mu$M genistein produced no significant change in $I_{sc}$ in the tracheas from CF null mice ($0.9 \pm 1.1 \mu A/cm^2$, $n = 9$) (Fig. 2, B and C). This is highly significantly different from the increase in $I_{sc}$ observed after addition of genistein to wild-type tracheas ($P < 0.0005$).

Genistein does not affect $Ca^{2+}$-mediated $Cl^-$ secretion. A cocktail of the calcium ionophore A-23187 (1 $\mu$M) and the $Ca^{2+}$-ATPase inhibitor TBHQ (25 $\mu$M) (26) elicited a further increase in $I_{sc}$ after addition of
genistein in both wild-type (56.4 ± 11.6 μA/cm², n = 8) and CF null mice (45.1 ± 9.1 μA/cm², n = 5) (Fig. 2). These responses are not significantly different from those observed when these drugs were added before genistein (data not shown). The subsequent addition of 10 μM forskolin in wild-type tracheas produced only a small increase in $I_{sc}$ (2.5 ± 1.2 μA/cm², n = 8) (Fig. 2, A and D). However, when the order of drug addition to a wild-type trachea was reversed after addition of genistein, the forskolin response was 20.5 ± 3.9 μA/cm² and the A-23187/TBHQ response was 39.3 ± 6.5 μA/cm² (n = 8) (Fig. 2D). The order of drug addition did not significantly alter the response to A-23187/TBHQ; however, the response to forskolin was significantly greater when the drug was added directly after genistein ($P < 0.001$) than when added after both genistein and A-23187/TBHQ (Fig. 2D). When genistein was added directly after forskolin to wild-type tracheas, the genistein produced no further increase in $I_{sc}$ (n = 5, data not shown).

The same effect was observed in CF null tracheas. The addition of forskolin, after both genistein and A-23187/TBHQ, produced no further increase in $I_{sc}$ (Fig. 2B), but when forskolin was added directly after genistein, it produced an increase in $I_{sc}$ of 29.5 ± 8.6 μA/cm² (n = 7), and the subsequent addition of A-23187/TBHQ produced a further increase of 34.7 ± 9.0 μA/cm² (n = 7). In wild-type and CF null tracheas the combined increase in $I_{sc}$ in response to both A-23187/TBHQ and forskolin was independent of the order of drug addition. In wild-type tracheas the total increase in $I_{sc}$ was 59.8 ± 8.5 μA/cm² after A-23187/TBHQ followed by forskolin (n = 8) and 56.4 ± 11.6 μA/cm² after forskolin followed by A-23187/TBHQ (n = 8). In CF null tracheas the increase in $I_{sc}$ was 46.4 ± 6.4 μA/cm² after A-23187/TBHQ followed by forskolin (n = 7) and 64.3 ± 17.2 μA/cm² after forskolin followed by A-23187/TBHQ (n = 7). These values are not significantly different.

Delivery of hCFTR cDNA to the airways of CF null mice restores the genistein response in the trachea. CF null mice were transfected with either a hCFTR expression plasmid (pT10CFTR) or empty plasmid vector (pT10) complexed with the liposome DC-Chol:DOPE. Two days after transfection the tracheas were mounted in Ussing chambers for $I_{sc}$ measurements. Tracheas transfected with pT10CFTR showed a genistein-sensitive increase in $I_{sc}$ of 11.2 ± 2.6 μA/cm² (n = 8) (Fig. 3, C and D), which is significantly higher than that in untreated CF null mice ($P < 0.002$) and not significantly different from that in wild-type. The trachea of CF null mice transfected with pT10 showed a small genistein-sensitive current of 4.2 ± 1.6 μA/cm² (n = 7) (Fig. 3D), which is not significantly different from that in untreated CF null tracheas and significantly smaller than that in tracheas of CF null mice treated with pT10CFTR ($P < 0.05$) and in untreated wild-type tracheas ($P < 0.01$). The responses to A-23187/TBHQ (CF null + pT10CFTR: 63.1 ± 10.6 μA/cm², n = 13; CF null + pT10: 46.4 ± 6.4 μA/cm², n = 7) and amiloride.
(CF null + pT10CFTR: −40.5 ± 9.6 μA/cm², n = 13; CF null + pT10: −26.6 ± 5.2 μA/cm², n = 8) were not significantly different between CF null mice transfected with either plasmid and untreated CF null mice.

Genistein is able to increase $I_{sc}$ responses in some tracheas taken from ΔF508 mice. The effect of genistein on tracheas taken from ΔF508 homozygous mice (Cftr<sup>tm2Cam</sup>) (4) was studied (Fig. 4). Individual tra-
cheas varied in their response to the addition of genistein. Forty-six percent showed no response or a very small response to 50 μM genistein (0.26 ± 0.6 μA/cm², n = 6) (Fig. 4A). However, some tracheas responded to genistein at levels similar to those seen in wild-type mice (12.0 ± 2.1 μA/cm², n = 7) (Fig. 4B). Overall, the mean response from all the DF508 tracheas studied was 7.1 ± 1.9 μA/cm² (n = 13) (Fig. 4C), significantly smaller than the genistein response in wild-type tracheas (P < 0.02). The responses to amiloride (−21.3 ± 3.3 μA/cm², n = 13) and A-23187/TBHQ (75.9 ± 12.4 μA/cm², n = 13) were not significantly different from those measured in either wild-type or CF null tracheas. Tracheas that did not respond to genistein showed amiloride and A-23187/TBHQ responses similar to those of tracheas that did demonstrate a genistein-induced increase in I_{sc} (data not shown).

The effect of genistein on I_{sc} responses of wild-type, CF null, and ΔF508 colons. The effect of genistein on the I_{sc} of colons from wild-type, CF null, and ΔF508 mice was measured (Fig. 5). Bilateral addition of 50 μM genistein (after amiloride) to wild-type colons resulted in an increase in I_{sc} of 21.0 ± 8.1 μA/cm² (n = 4). This is significantly smaller than the increase induced by 10 μM forskolin (146.0 ± 21.9 μA/cm², n = 8). Further addition of 50 μM genistein to give a final concentration of 100 μM resulted in a further increase in I_{sc} of 27.4 ± 10.8 μA/cm² (n = 4). The combined I_{sc} increase of genistein and forskolin was 75.3 ± 11.4 μA/cm², which is signif-

Fig. 5. The effect of genistein on colons from wild-type, CF null, and ΔF508 mice. A: changes (mean ± SE) in I_{sc} following drug addition in colons taken from wild-type, CF null, and ΔF508 mice. Drugs were added at the following concentrations: amiloride, 100 μM, apical membrane only; genistein, 100 μM, apical and basolateral membranes; forskolin, 10 μM, apical and basolateral membranes; furosemide, 1 mM, basolateral membrane only. The basal I_{sc} and response to amiloride is significantly different in wild-type (n = 8) and ΔF508 colons (n = 9). The response to forskolin is significantly smaller in CF null colons (n = 10) compared with ΔF508 colons (n = 9). B: a comparison of the change in I_{sc} in response to forskolin (10 μM) only or to genistein (100 μM) followed by forskolin (10 μM) in wild-type, CF null, and ΔF508 colons. Amiloride (100 μM) was added first in all experiments. Values are means ± SE; wild type: n = 8; CF null: n = 21 (forskolin) and n = 10 (genistein + forskolin); ΔF508: n = 10 (forskolin) and n = 11 (genistein + forskolin). P values are indicated where significant differences were observed.
resulted in a significant decrease (P < 0.02) (Fig. 5B). Furosemide inhibited this increase by 63.2 ± 5.2% (n = 8), and DPC inhibited the remaining current by 71.8 ± 26.6% (n = 4).

In colons from CF null and ΔF508 mice, genistein caused a decrease in $I_{sc}$ in all tissues tested (CF null: $-13.1 ± 2.3 \mu A/cm^2$, n = 10; ΔF508: $-10.3 ± 1.3 \mu A/cm^2$, n = 9), and 10 μM forskolin produced a further decrease of $-6.5 ± 1.2 \mu A/cm^2$ in CF null colons (n = 10) and $-14.0 ± 3.0 \mu A/cm^2$ in ΔF508 colons (n = 9) (Fig. 5A). Forskolin alone decreased $I_{sc}$ by $-24.8 ± 1.5$ (CF null, n = 21) or $-17.2 ± 1.5 \mu A/cm^2$ (ΔF508, n = 10). In both CF null and ΔF508 colons, the combined change in response to genistein and forskolin was not significantly different from the change in response to forskolin alone (Fig. 5B). For both genotypes furosemide completely reversed the change in $I_{sc}$ due to genistein and forskolin (Fig. 5A). The response to genistein was similar in CF null and ΔF508 colons, but the response to forskolin after genistein was significantly larger in ΔF508 colons (Fig. 5A, P < 0.05), although the response to forskolin alone is significantly smaller in ΔF508 colons compared with CF null colons ($-17.2 ± 1.5 \mu A/cm^2$, n = 10 vs. $-24.8 ± 1.5 \mu A/cm^2$, n = 21; P < 0.005) (Fig. 5B). However, the ΔF508 colon response to forskolin after genistein is not significantly different from the ΔF508 responses to forskolin with no prior genistein.

Both the basal $I_{sc}$ ($17.1 ± 4.2 \mu A/cm^2$, n = 9) and the amiloride-sensitive $I_{sc}$ ($-5.0 ± 0.5 \mu A/cm^2$, n = 9) of ΔF508 colons were significantly smaller than those measured in wild-type colons (basal $I_{sc}$: $27.0 ± 3.9 \mu A/cm^2$; amiloride-sensitive $I_{sc}$: $-10.8 ± 3.1 \mu A/cm^2$, n = 8; P < 0.05) (Fig. 5A).

**DISCUSSION**

Others have shown that genistein can stimulate CFTR Cl- channels in a variety of cell systems (8, 14–16, 18, 20, 21, 31, 32, 41). Here we report the first description of the action of genistein on intact murine tissues. Genistein, but not genistin, increased $I_{sc}$ when added to either wild-type tracheal or colonic tissues. A number of experiments were conducted to confirm that this response was due to CFTR-mediated Cl- secretion.

First, removal of Cl- from the bathing buffer resulted in a significant decrease (P < 0.01) in the $I_{sc}$ response to the addition of genistein. The residual small genistein-sensitive $I_{sc}$ may be due to HCO$_3^-$ secretion through CFTR (20, 25, 37). Second, the genistein response was inhibited by DPC and glibenclamide but not by DIDS. DPC is a nonspecific Cl- channel inhibitor that has been shown to inhibit CFTR (2), whereas the sulfonylurea glibenclamide inhibits CFTR at 100 but not 1 μM (29, 34). However, glibenclamide also inhibits the outwardly rectifying Cl-channel (ORCC) at 100 μM (29). Although it is able to block the ORCC at an extracellular concentration of 10 μM, up to 500 μM extracellular DIDS has no effect on the activity and conductance of CFTR (10). Thus the insensitivity of the genistein response to 300 μM DIDS is characteristic of CFTR-mediated Cl- secretion. Third, the genistein response was inhibited by furosemide, suggesting that the genistein-mediated increase in $I_{sc}$ was due to Cl- secretion. Finally, when genistein was tested on tracheas taken from CF null mice, which have no functional CFTR, no response was observed. The response to genistein could be restored to wild-type levels by transfecting the tracheas of CF null mice with a plasmid expressing hCFTR. We conclude from these results that genistein activates Cl- secretion through CFTR in the murine trachea, although we cannot formally rule out the small possibility that Cl- secretion is through an alternative Cl- channel whose opening depends on the presence of CFTR. However, this latter possibility is considered unlikely because it has been shown by patch-clamp analysis that genistein activates CFTR in cell systems.

Current data suggest that one mechanism of action of genistein is by direct binding to CFTR to promote channel opening, and this requires the presence of ATP and phosphorylation of the R domain (8, 40, 41). It is interesting to note that in our tracheal studies, prior treatment with a submaximal concentration of forskolin to stimulate CFTR phosphorylation did not augment the genistein response. This is in contrast to studies showing that genistein required the prior addition of low concentrations of forskolin (1 μM) to reach the same levels of stimulation seen with 10 μM forskolin. In our study, prior addition of low concentrations of forskolin (100 nM) or IBMX (1 μM) did not increase the response to genistein, suggesting that the basal phosphorylation state of CFTR in the murine trachea is sufficient to obtain the maximum activation possible by 50 μM genistein under the conditions studied. Thus there may be a significant difference between cell systems and fresh tissue in the extent to which CFTR is phosphorylated at the basal level. Illeck et al. (17) have also stated that in the presence of amiloride and/or low-Cl- buffer, genistein is able to stimulate the in vivo nasal potential difference of humans without the prior addition of isoproterenol. When the prior addition of higher levels of forskolin (1 μM) or IBMX (100 μM) was tested, it was found that genistein could produce no additional increase in $I_{sc}$. Thus further elucidation of the combined actions of these drugs to try and understand their mechanism of action is probably limited by the total Cl- secretory capacity of the tracheal epithelium.

The data support the observation that the response to forskolin is likely to be composed of both cAMP- and Ca$^{2+}$-mediated Cl- secretion (12, 23), whereas the genistein response is mediated only by CFTR Cl- secretion. In our study, forskolin added immediately after genistein further increased $I_{sc}$, whereas forskolin added after genistein and A-23187/TBHQ produced no further increase in $I_{sc}$. The addition of A-23187/TBHQ to the epithelium should result in maximal activation of Ca$^{2+}$-mediated Cl- secretion because A-23187 allows free entry of Ca$^{2+}$ into the cell and TBHQ empties internal stores of Ca$^{2+}$ and prevents them from refil-
ing (28). Our data suggest that genistein has maximally activated CFTR and that A-23187/TBHQ have maximally stimulated Ca\(^{2+}\)-mediated Cl\(^{-}\) secretion, allowing no further stimulation by forskolin. The \(I_{sc}\) response to forskolin when it is added directly after genistein may be due to forskolin stimulating Ca\(^{2+}\)-mediated Cl\(^{-}\) secretion rather than cAMP-mediated Cl\(^{-}\) secretion.

Tracheas taken from ΔF508 mice showed a bimodal response to genistein, eliciting either no increase in \(I_{sc}\) or a secretory response similar to that in wild type. The ΔF508 mice (like the CF null mice) are maintained as an outbred stock (129Sv/Ev × MF1). It is possible that particular combinations of modifier genes might allow trafficking of the ΔF508 protein to the apical membrane in some mice, where the channel can be opened by genistein (14, 19). In mice that did not respond to genistein, one would assume that no ΔF508 protein had reached the apical membrane. Unfortunately, because of the very low amounts of CFTR protein in the tracheal epithelium, we have been unable to detect either wild-type or ΔF508 protein in cells of the tracheal epithelium by immunohistochemistry (Ratcliff R, personal communication), so further electrophysiological techniques are required to ascertain whether ΔF508 protein can reach the tracheal epithelium apical membrane in a subset of ΔF508 mice.

In the trachea, 50 μM genistein was able to elicit an increase in \(I_{sc}\) similar to that elicited by 10 μM forskolin; however, in the colon, 100 μM genistein did not increase \(I_{sc}\) to the same level as forskolin. This could be because the basal level of phosphorylation of CFTR is less in the colonic epithelium than in the tracheal epithelium. Consistent with this possibility, Diener and Hug (6) demonstrated that prior addition of 200 nM forskolin to the rat distal colon enhanced the genistein response. In our study the addition of forskolin after genistein treatment produced a further increase in \(I_{sc}\) similar in size to the initial increase induced by genistein. However, the total increase in \(I_{sc}\) due to genistein and forskolin is still lower than the increase generated by forskolin alone. Diener and Hug (6) concluded that genistein interacts in synergy with the cAMP-mediated Cl\(^{-}\) secretion of the colon but antagonistically with Ca\(^{2+}\)-dependent K\(^{+}\) conductance. They showed that genistein inhibits carbachol-mediated K\(^{+}\) conductance. This would explain why the genistein and forskolin \(I_{sc}\) responses are lower than expected, because if genistein inhibits basolateral K\(^{+}\) conductance, this will reduce the driving force for Cl\(^{-}\) secretion across the apical membrane. Also, it is clear from the results of the CF null and ΔF508 colons that genistein activates cation secretion, and this will appear to reduce the total anion secretion. From the data we conclude that genistein activates Cl\(^{-}\) secretion through CFTR in the wild-type colon because the increase in \(I_{sc}\) was inhibited by furosemide and DPC and because no Ca\(^{2+}\)-dependent Cl\(^{-}\) channel is present in the apical membrane of the distal colon epithelium (3).

Genistein decreased \(I_{sc}\) when added to the colon from CF null and ΔF508 mice. This was followed by a further decrease in response to forskolin in both genotypes, and the combined decrease in current was not significantly different from the decrease in \(I_{sc}\) caused by forskolin alone. This finding suggests that genistein is activating the same K\(^{+}\) secretion pathway as forskolin (5). There was also no difference in the overall response to genistein and forskolin in the CF null and ΔF508 colons, although the response to forskolin after genistein addition was significantly greater in the ΔF508 colon compared with the CF null colon. The simplest explanation is that this is due to the tendency for the ΔF508 colon to show a smaller response to genistein than CF null colon, resulting in the ability of forskolin to have a larger effect before the tissue reached its maximum response.

The basal \(I_{sc}\) of ΔF508 colons, although not the basal \(I_{sc}\) of CF null colons, was significantly smaller than that of wild-type colons. This was due to hypoabsorption of Na\(^{+}\), because the amiloride-sensitive current was less in ΔF508 colons than in wild-type colons. This result differs from that of a previous report by Grubb and Boucher (11), which showed that distal colon from the Cftr\(^{tm1UNC}\) CF null mouse had a significantly lower basal \(I_{sc}\) than wild-type tissue because of a lack of Cl\(^{-}\) and HCO\(_{3}^{-}\) secretion. Also, the distal colon of wild-type mice in the Grubb and Boucher study had an approximately fourfold higher basal \(I_{sc}\) compared with the wild-type mice used in this study, and neither wild-type nor Cftr\(^{tm1UNC}\) tissue showed significant amiloride sensitivity under normal dietary conditions. The different results of the two studies could reflect differences in the level and/or regulation of the relevant ion channels due to the different genetic backgrounds of the two stocks. Interestingly, our study also found that there was no difference in the amiloride sensitivity of tracheas from wild-type, CF null, and ΔF508 mice, unlike human CF airway tissue, which normally demonstrates hyperabsorption of Na\(^{+}\). This finding suggests that the interaction of CFTR and ENaC may be different between mice and humans and that in the tracheal epithelium of mice from Cftr\(^{tm1Cam}\) and Cftr\(^{tm2Cam}\) stocks, CFTR does not act as a negative regulator of ENaC.

In conclusion, we have demonstrated that genistein can increase \(I_{sc}\) in wild-type murine tracheas and colons and some ΔF508 tracheas but not in CF null murine tracheas or CF null and ΔF508 colons. Together with the data from Cl\(^{-}\) channel blockers and low-Cl\(^{-}\) buffer, the results strongly suggest that the increase in \(I_{sc}\) is due to Cl\(^{-}\) secretion through CFTR. The lack of a genistein-sensitive \(I_{sc}\) in CF null tracheas compared with the wild type provides an alternative procedure to distinguish the two genotypes that is more selective than forskolin, which also activates Ca\(^{2+}\)-mediated Cl\(^{-}\) secretion. This allows CF null tracheas to be used from mice of any age to test strategies to restore a CFTR channel to the tracheal epithelium. The genistein-sensitive \(I_{sc}\) seen in some ΔF508 tracheas suggests that these mice may be useful for studying the action of genistein and other flavonoids on ΔF508 protein, which may lead to clinical applications.
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