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Genistein Modifies the Activation Kinetics and Magnitude of Phosphorylated Wild-Type and G551D-CFTR Chloride Currents

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Abstract. We have studied the mechanism by which genistein activates cystic fibrosis transmembrane conductance regulator (CFTR) in CHO cells expressing wild type or G551D-CFTR. In wild-type CHO cells, after exposure to 2.5 μM forskolin, 25 μM genistein induced a further 2-fold and rapid increase of the forskolin-activated CFTR current. In both types of cells, when forskolin was added after genistein preincubation, whole-cell current density was greatly reduced compared to that measured when genistein was added after phosphorylation of CFTR, and all activation kinetic parameters were significantly altered. Genistein had no effect on the adenylate cyclase activity. Our results suggest that the occupancy of a putative genistein binding site is critical for the gating mechanism of CFTR chloride channels, which, depending on the phosphorylation status of the R-domain, drives CFTR either into a refractory state or alternatively to a highly activated state.

Key words: Cystic fibrosis transmembrane conductance regulator (CFTR) — Whole-cell recording — Pharmacology — Genistein — Phosphorylation

Introduction

The Cystic Fibrosis Transmembrane conductance Regulator (CFTR) is an epithelial cAMP-dependent Cl^- channel whose regulation involves phosphorylation by protein kinase A (PKA) and C (PKC) on serine residues located in the intracellular regulatory (R) domain, and subsequent hydrolysis of ATP at two nucleotide binding domains, NBD1 and NBD2 (for review, *see* Sheppard & Welsh, 1999). CFTR mutations in the genetic disease cystic fibrosis (CF) either eliminate or markedly impair this conductance pathway. Among the 900 mutations associated with a CF disease phenotype, the G551D mutation is the third most common one with a frequency of 2–5%, depending on the population of origin (Hamosh et al., 1992; Cashman et al., 1995). The mutated protein is correctly inserted in the apical membrane, but its channel activity is strongly reduced (Welsh & Smith, 1993). G551D, localized in NBD1 of CFTR, interferes with ATP binding (Logan et al., 1994) and hydrolysis (Li et al., 1996).

A number of compounds have been shown to activate CFTR by mechanisms that do not involve an increase of intracellular cAMP, including the isoflavone genistein, originally known as an inhibitor of protein tyrosine kinases (PTKs). Since the first report by Illek et al. (1995), genistein has been widely used to study cAMP-independent activation of CFTR in various cell systems, including T84 and HT29 intestinal cells (Illek, Fischer & Machen, 1996), human airway Calu-3 cells (Illek & Fischer, 1998), cardiac myocytes (Obayashi et al., 1999) and epididymal epithelium cells (Leung & Wong, 2000).

Initial studies proposed that genistein stimulates channel activity by preventing the inhibition of CFTR by protein tyrosine kinase (PTK) (Illek et al., 1995; Sears et al., 1995), but more recent reports indicated that genistein affects CFTR-mediated ion transport by direct interaction with the channel (French et al., 1997; Wang et al., 1998; Lansdell et al., 2000). Consistent with this idea, it was demonstrated that the binding of genistein to a recombinant NBD2 protein inhibits the ATPase activity of NBD2 (Randak et al., 1999), whereas no effect of genistein was detected on the ATPase activity of NBD1 (Howell,

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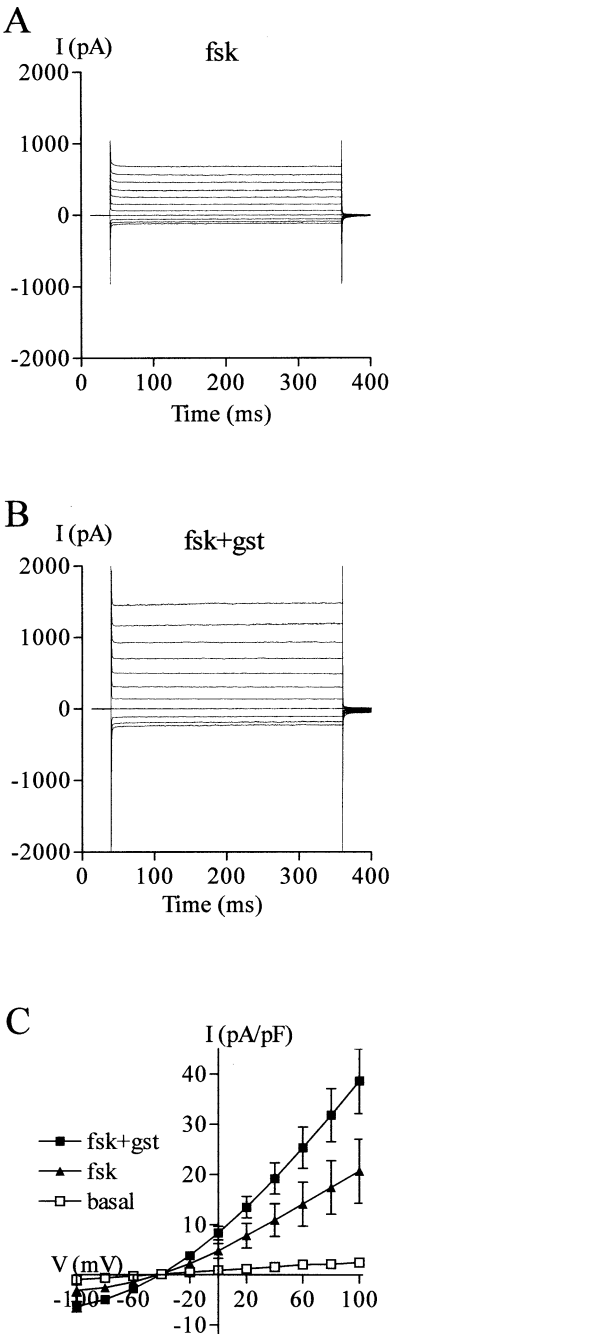

Fig. 1. Effect of genistein on forskolin-stimulated CFTR Cl^- channels. Representative current traces for CFTR in CFTR(+) CHO cells. Current was recorded with 2.5 μM forskolin (*A*) in the bath. After 3 minutes, 25 μM genistein was added (*B*). Protocol consisted of 300-msec voltage steps ranging from −100 to +100 mV from a holding potential of −40 mV. Capacitance of cell was 21 pF. (*C*) Corresponding *I–V* curves (mean ± SEM, $n = 8$) for CFTR current.

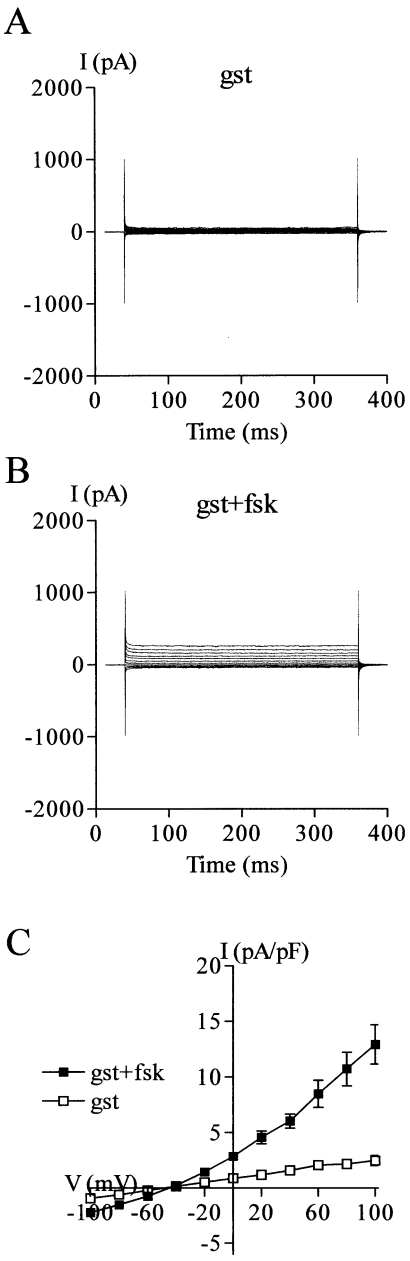

Fig. 2. Effect of forskolin on wild-type CFTR Cl^- channel preincubated with genistein. Representative current traces for CFTR. No current was recorded with 25 μM genistein (*A*) in the bath. After 3 minutes, 2.5 μM forskolin was added (*B*). For stimulation protocol, *see* Fig. 1. Capacitance of cell was 23 pF. (*C*) Corresponding *I–V* curves (mean ± SEM, $n = 5$) for CFTR current.

Borchardt & Cohn, 2000). In most studies, the effect of genistein was only seen on the CFTR Cl^- conductance after prior phosphorylation of the channel (Reenstra et al., 1996; Yang et al., 1997). Further, genistein failed to elevate intracellular cAMP (Illek et al., 1995; Sears et al., 1995). Based on these and other results it was proposed that genistein inhibited CFTR dephosphorylation (Illek et al., 1996; Reenstra et al., 1996; Yang et al., 1997). Luo et al. (2000) finally showed that genistein is not a phosphatase inhibitor, pointing to a more direct effect on CFTR; indeed, Wang et al. (1998) speculated that genistein binding to CFTR locks the channel in the open configuration, which greatly increases the channel opening duration.

Table 1. Comparison of time constants determined at different conditions of activation in wild-type CHO cells

Recording configuration	First activator	Second activator	Activation delay			Time constant $1/K$		
			sec	n	P	sec	n	P
a) Whole-cell	Fsk	None	33 ± 3	7		40 ± 20	7	
b) Whole-cell	Fsk	Gst	5.0 ± 0.4	4	**(a)	2 ± 2	4	**(a)
c) Whole-cell	Gst	Fsk	83 ± 9	3	**(a, b)	46 ± 33	3	*ns* (a) * (b)
d) Perforated patch	Gst	Fsk	77 ± 12	3	**(a) *ns* (c)	20 ± 17	3	*ns* (a, c)

The activation delay corresponds to the time between activator addition and beginning of current increase. The time constant $1/K$ was obtained by fitting the time-course data (*see* Fig. 3) with an exponential function, $[\Delta I \cdot (1-\exp(-K \cdot T)) + I_{basal}]$. Values are mean ± SEM. * indicates significantly different with $0.01 < P < 0.05$; ** indicates significantly different with $P < 0.01$; *ns*: not significantly different. Statistical differences for data in a given row were determined compared to the row(s) indicated in parentheses.

In the present study, we further explored the mechanism by which genistein activates wild type and G551D-CFTR expressed in CHO cells. We focused on interaction between the phosphorylation level of CFTR and its activation by genistein.

Materials and Methods

Cell Culture

Chinese Hamster Ovary cells, stably transfected with pNUT vector containing wild-type CFTR (CFTR(+) CHO) or G551D mutation (G551D CHO) were provided by J.R. Riordan and X.-B. Chang, Scottsdale, AZ, USA (Tabcharani et al., 1991). Cells cultured at 37°C in 0.5% CO_2 were maintained in αMEM containing 7% fetal bovine serum, 0.5% L-glutamine, 0.5% antibiotics (50 IU/ml penicillin and 50 μg/ml streptomycin) and 100 or 20 μM methotrexate for, respectively, CFTR(+) or G551D CHO. For detailed procedures *see* Tabcharani et al. (1991).

Current Recording

Whole-cell recordings were performed on CHO cells plated on 35-mm petri dishes and cultured at 37°C in 5% CO_2. Currents were recorded with a List EPC-7 patch clamp amplifier, filtered at 3 kHz and digitized at 40 kHz, as reported elsewhere (Bulteau et al., 2000). The membrane potential was clamped to −40 mV and pulsed from −100 to +100 mV by 20-mV steps. Pipettes with resistances of 3–4 MΩ were pulled from borosilicate glass capillary tubing (GL150-T10, Clark Electromedical, Reading, UK) using a two-step vertical puller (Narishige, Japan). They were connected to the head stage of the amplifier through an Ag-AgCl pellet. Seal resistances ranging from 10 to 20 GΩ were obtained. The pipette solution contained (in mM): 113 L-aspartic acid, 113 CsOH, 27 CsCl, 1 NaCl, 1 EGTA, 3 MgATP and 10 TES, pH 7.4, 285 mOsm. The external solution consisted of (in mM) 145 NaCl, 4 CsCl, 1 $MgCl_2$ 1 $CaCl_2$, 5 glucose, 10 TES and 30 mannitol, pH 7.4, 340 mOsm. Mannitol was added to the external solution to avoid activation of swelling-induced chloride conductance. For the perforated-patch technique, amphotericin-B was freshly dissolved in DMSO and then diluted in electrode filling solution to give a final amphotericin concentration of 150 μg/ml. The tip of the pipette was dipped into amphotericin-free solution, the pipette was then backfilled with ionophore, and a gigaseal was formed as rapidly as possible. Results were analyzed with the pCLAMP6 package software (pCLAMP, Axon Instruments, Foster City, CA). All experiments were performed at room temperature. Cells were stimulated with appropriate compounds at the concentrations indicated in the text (dissolved in dimethylsulfoxide DMSO; final DMSO concentration: 0.1%). In control experiments, the currents were not altered by DMSO.

Membrane Preparation and Adenylate Cyclase Assay

CHO cells were homogenized with a Potter homogenizer in a hypotonic buffer (1 mM Tris-HCl, 1 mM $MgCl_2$ and 1 mM EGTA, pH 7.4). Crude membranes were obtained by centrifugation at 25,000×*g*, 5 min at 4°C and assayed immediately for adenylate cyclase activity (Johnson & Salomon, 1991). Briefly, crude membranes (20–50 μg of protein) were incubated at 32°C for 10 min

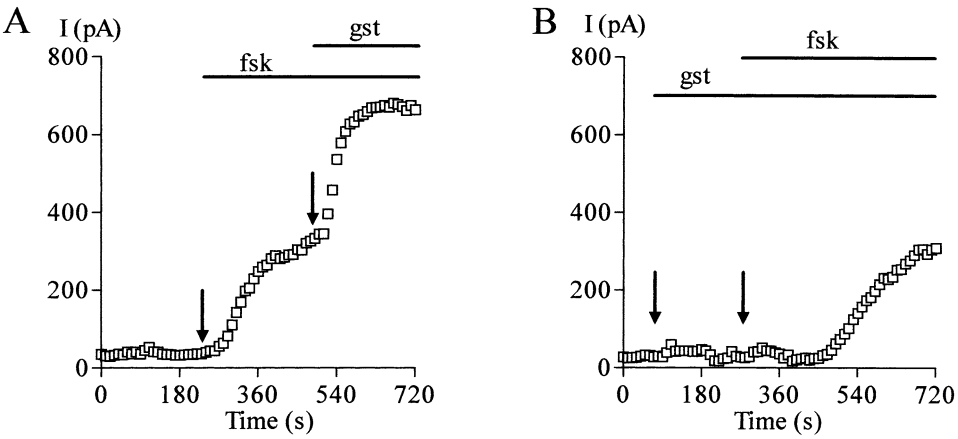

Fig. 3. Time course of genistein-activated CFTR current in CFTR(+) CHO cells. Whole-cell current at +40 mV is plotted as function of time in presence of 2.5 μM forskolin, and 25 μM genistein, as shown. (*A*) Forskolin is first activator. (*B*) Forskolin is second activator. Arrows denote time of activator application.

with test compounds in a solution containing (in mM) 20 Tris-HCl (pH 7.4), 0.5 IBMX, 2 creatine phosphate, 0.5 ATP, 4 $MgCl_2$, 200 μg/ml creatine kinase, and 0.1% bovine albumine. The reaction was stopped by adding 12% trichloroacetate acid (TCA). Following centrifugation, TCA was removed with water-saturated diethylether and cAMP was assayed by means of radioimmunoassay (Amersham Pharmacia Biotech, UK).

Chemicals

All products were from Sigma (St Louis, MO) except αMEM, which was from Fisher PAA.

Data Analysis

All values are presented as means ± SEM. Mean current densities were expressed in pA/pF to account for differences in cell membrane area. Significance was determined using a Student's *t*-test for unpaired data. A value of $P < 0.05$ was assumed to indicate a statistically significant difference.

Results

We compared activation of wild type and G551D-CFTR by low concentrations of forskolin and genistein, with two protocols of activation, using the whole-cell and perforated patch-clamp techniques. In the first protocol, cells were preincubated during 3 minutes with 2.5 μM (for CFTR(+) CHO) or 10 μM (for G551D CHO) forskolin, then 25 μM genistein was added in the presence of forskolin. In the second approach, cells were first incubated with 25 μM genistein, then forskolin was added in the presence of genistein.

Figure 1 shows representative traces from a wild-type CHO cell, which was prestimulated with forskolin (2.5 μM, Fig. 1*A*). Subsequent addition of genistein (25 μM, Fig. 1*B*) stimulated a large Cl^- current in 8/11 (73%) cells, which was time- and voltage-independent. The genistein-activated Cl^- conductance (Fig. 1*C*) had a current density of 19.2 ± 3.1 pA/pF ($n = 8$) when measured at +40 mV. The increase was significantly different from the basal current ($P < 0.001$). Because both currents activated by forskolin or forskolin plus genistein reversed at −40 mV, a potential close to the Cl^- equilibrium potential determined by our conditions (−37 mV), and since they were not blocked by 200 μM DIDS, but completely inhibited by 100 μM glibenclamide (*data not shown*), we concluded that the forskolin- or genistein-activated Cl^- currents are supported by CFTR.

When 25 μM genistein alone was applied in the bath (Fig. 2*A*), little or no current was activated. Subsequent addition of forskolin in the presence of genistein (Fig. 2*B*) stimulated Cl^- current only in 5/17 (29%) cells tested. Current amplitudes were very weak, with a current density of 6.4 ± 0.9 pA/pF ($n = 5$) (basal current 1.8 ± 0.5 pA/pF, $n = 5$) when measured at +40 mV (Fig. 2*C*). The maximal amplitude of the current was significantly lower (3-fold) when forskolin was applied after ($P < 0.01$) than when it was applied before genistein treatement.

A time course of the activation of CFTR current for the different conditions is shown in Fig. 3. Addition of genistein after forskolin treatment (Fig. 3*A*) led to an immediate (within 5.0 ± 0.4 sec, *see* Table 1) and fast current increase (time constant to maximal activation 42 ± 2 sec, Table 1), both significantly different from activation with forskolin alone (33 ± 3 sec and 140 ± 20 sec, respectively, Table 1). On the contrary, when genistein was first applied, the subsequent addition of forskolin (Fig. 3*B*) induced a significantly delayed current increase, compared to experiments using forskolin alone or genistein added after forskolin (Table 1). This was in agreement with iodide efflux experiments (*data not shown*), where the peak relative rates were lower when

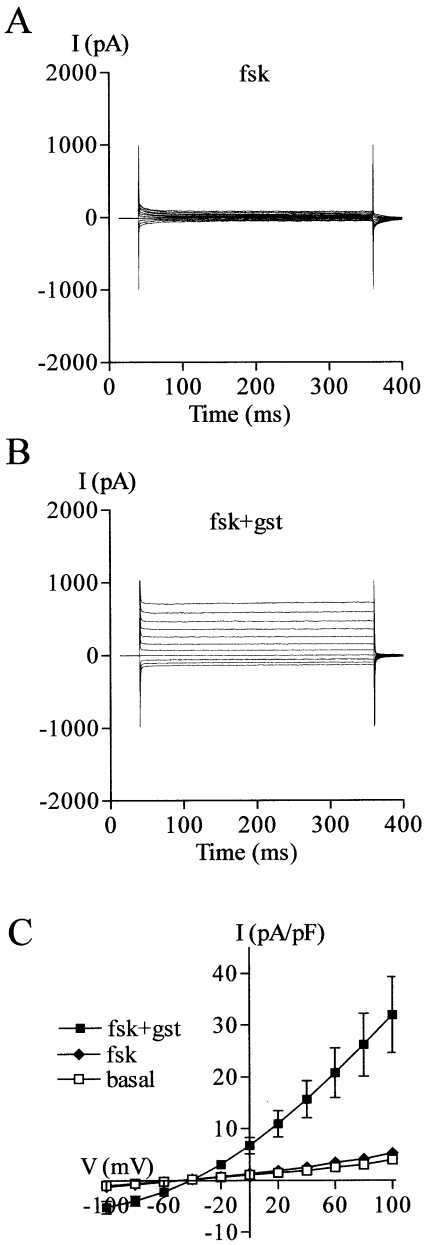

Fig. 4. Effect of genistein on forskolin-stimulated G551D-CFTR Cl^- channels. Representative current traces for CFTR. Current was recorded with 10 μM forskolin (*A*) in the bath. After 3 minutes, 25 μM genistein (*B*) was added. For stimulation protocol, *see* Fig. 1. Capacitance of cell was 27 pF. (*C*) Corresponding *I–V* curves (mean ± SEM, $n = 16$) for CFTR current.

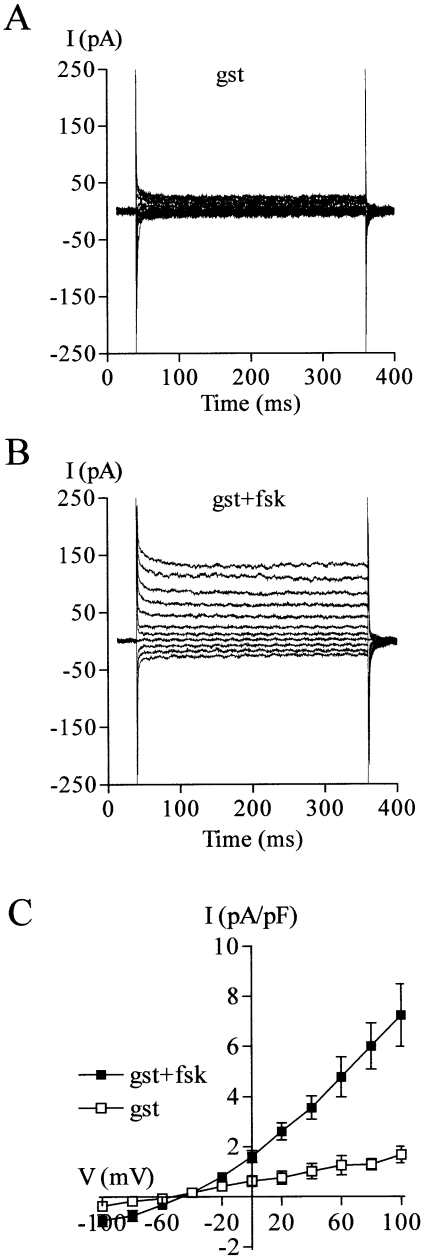

Fig. 5. Effect of forskolin on genistein-stimulated G551D-CFTR Cl^- channels. Representative current traces for CFTR for cell first incubated with 25 μM genistein (*A*), then in the presence of 10 μM forskolin (*B*). For stimulation protocol, *see* Fig. 1. Capacitance of cell was 14 pF. (*C*) Corresponding *I–V* curves (mean ± SEM, $n = 3$) for CFTR current. Note the different current amplitude scale compared to Fig. 4.

cells were first preincubated with genistein (2.6 + 0.4 *vs.* 3.2 ± 0.2 without preincubation of genistein, $P < 0.01$). Since the soluble contents of the cell dialyze out of the cell in whole-cell recordings, the delay in current activation with the second protocol could be due to the loss of CFTR-associated PKA. To test this hypothesis, perforated patch-clamp experiments were performed (Table 1). However, as in whole-cell configuration, addition of forskolin after preincubation with genistein led to a delayed current activation (*compare* lines *c* and *d* to lines *a* and *b* in Table 1), invalidating the cell dialysis hypothesis.

Since one of the main differences between wild-type and G551D-CFTR is the lack of responsiveness of the mutant to cAMP agonists, we then performed the same study in G551D CHO cells (Fig. 4). No current was activated in the presence of 10 μM fors-

Table 2. Comparison of time constants determined at different conditions of activation in G551D CHO cells

First activator	Second activator	Activation delay			Time constant $1/K$		
		sec	n	P	sec	n	P
Fsk	Gst	8 ± 1	5		138 ± 30	5	
Gst	Fsk	120 ± 40	3	*	522 ± 39	3	**

For methods of analysis, *see* Table 1. Values are mean ± SEM. *: significantly different from fsk + gst values, $0.01 < P < 0.05$; **: $P < 0.01$.

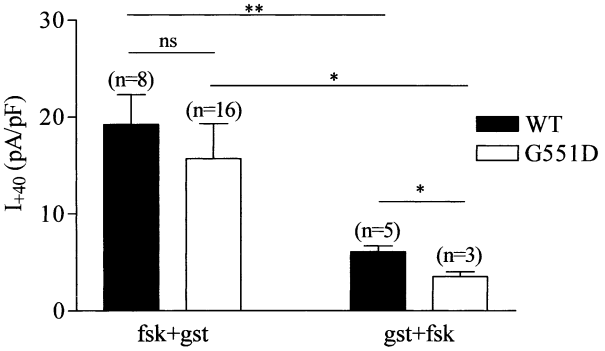

Fig. 6. Comparison of wild-type and G551D-CFTR current densities at +40 mV holding potential. Error bars are SEM; *P*, significant difference as indicated: $*0.01 < P < 0.05$; $**P < 0.01$; *ns*: no significant difference.

kolin (Fig. 4*A*), as was already shown by others (Illek et al., 1999). Subsequent addition of 25 μM genistein stimulated a Cl^- current in 16/26 (61% , Fig. 4*B*) cells. The genistein-activated Cl^- conductance (Fig. 4*C*) had a current density of 15.7 ± 3.6 pA/pF (n = 16) when measured at +40 mV. In G551D CHO cells not treated with forskolin (Fig. 5), genistein failed to stimulate Cl^- current (Fig. 5*A*). However, subsequent addition of 10 μM forskolin in the bath activates Cl^- current (Fig. 5*B*) in only 3/19 (16%) cells tested. This percentage of activation is close to that found for wild-type CFTR (*see* Fig. 2*B*). Current amplitude was very weak with a density of 3.5 ± 0.5 pA/pF, n = 3, when measured at +40 mV. Figure 6 summarizes and compares current densities recorded in both conditions. When genistein was first added to the bath, subsequent addition of forskolin led to a current density ~3-fold (for wild type) and ~4-fold (for G551D) lower than when CFTR was first stimulated by forskolin.

A time course of the activation of G551D-CFTR current is shown in Fig. 7. When genistein was first applied, subsequent addition of forskolin induced a significantly delayed current increase compared to results when genistein was added after forskolin (120 ± 40 *vs.* 8 ± 1 sec, Table 2). These values are not significantly different from those observed for wild-type CFTR (*compare* with Table 1). The genistein-activated current is slower (time constant 522 + 39 *vs.* 138 ± 30 sec, Table 2) when genistein was first added in the bath. Activation for G551D-CFTR current (for both conditions) is significantly slower than for wild-type CFTR (*compare* time constants in Tables 1 and 2).

Since genistein is thought to interact with a consensus sequence present in various ATP-dependent enzymes including topoisomerase II (Markovits et al., 1989), and was shown to inhibit protein tyrosine kinases and other ATP-requiring enzymes, the weak effect of forskolin, when genistein was added before forskolin, could be due to a competition between both compounds on adenylate cyclase. To test this hypothesis, we measured the adenylate cyclase activity of membrane preparations of CFTR(+) CHO cells (Fig. 8). When added in the presence of forskolin, genistein did not decrease forskolin activation of adenylate cyclase at either concentration used (31.3 ± 2.6 pmol/min/mg prot, n = 6, with forskolin *vs.* 33.1 ± 0.9 pmol/min/mg prot, n = 6, and 26.8 ± 2.3, n = 6, with forskolin plus 25 μM or 50 μM genistein, respectively).

Discussion

The isoflavone compound genistein has been shown to either stimulate (for concentrations below 50 μM) (Hwang & Sheppard, 1999) or inhibit (for high concentrations) (Wang et al., 1998; Lansdell et al., 2000) CFTR channel. Both types of studies were performed on phosphorylated CFTR protein. Here we show that low concentration of genistein (25 μM) differentially activates wild type or G551D-CFTR channels depending on whether the protein is phosphorylated before or after genistein interaction with CFTR.

Especially, it appears that preincubation of cells with genistein led to a modification of parameters of subsequent PKA-dependent activation of CFTR. When forskolin was added after genistein preincubation, (1) whole-cell current density was reduced by ~ 3-fold (for wild type) and ~4-fold (for G551D), (2) time to activation was greatly delayed, and (3) time constant of activation was reduced when compared with genistein activation after phosphorylation of CFTR.

Direct interaction of genistein with CFTR has been shown by Randak et al. (1999) who demonstrated

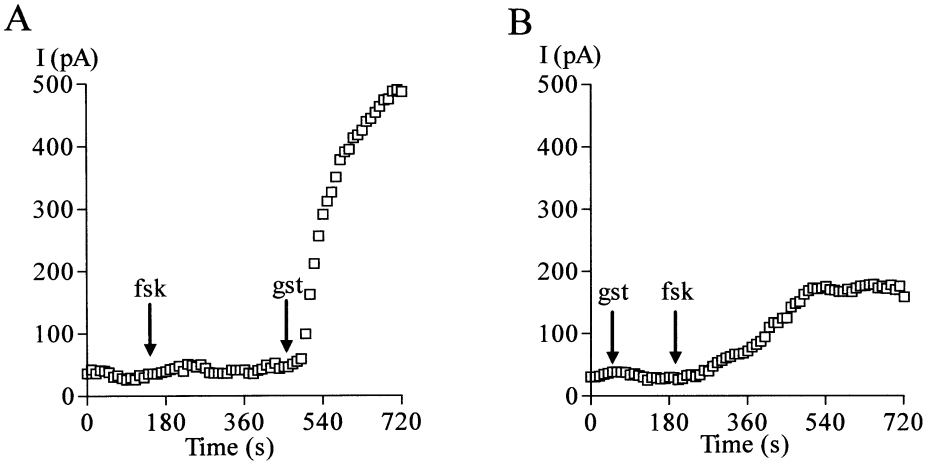

Fig. 7. Time-course of genistein-activated CFTR current in G551D CHO cells. Whole-cell current at +40 mV is plotted as function of time in presence of 10 μM forskolin and 25 μM genistein, as shown. (*A*) Application (arrows) of first forskolin, then genistein and (*B*), first genistein then forskolin.

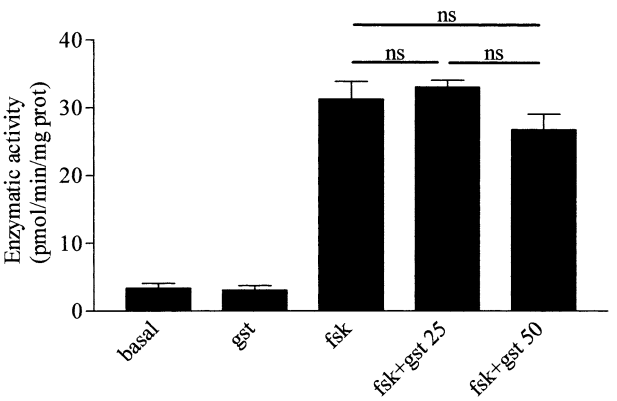

Fig. 8. Effect of genistein on adenylate cyclase activity in resting CFTR(+) CHO cells and during stimulation with forskolin (5 μM) or/and genistein (25 and 50 μM). Results are means ± SEM for $n = 6$. *t*-test: *ns*, not significantly different.

a binding of genistein to recombinant NBD2. To explain the delay in activating CFTR current with forskolin when cells were first preincubated with genistein, it can be hypothesized that conformational change after genistein binding on either NBD (probably NBD2) domain may impede access to phosphorylation sites. This raises the possibility that a part of NBDs may interact with the R domain, where PKA-dependent phosphorylation mainly occurs. In support of this, Wang et al. (2000) recently showed that deletion of phenylalanine 508 causes attenuated PKA-dependent activation of CFTR in stably transfected NIH3T3 cells. The authors concluded that there could be a functional perturbation in the R domain secondary to a structural change in NBD1. Therefore, in absence of prior phosphorylation, genistein could bind to NBD2, leading to an interdomain R-NBD interaction that impedes phosphorylation of the R domain and subsequent opening of the channel pore. Alternatively, it may be the response to phosphorylation that is affected by genistein, rather than phosphorylation itself. Indeed, no change in phosphopeptide maps was noticed in an in vivo phosphorylation study of CFTR after genistein (Reenstra et al., 1996). In addition, in our cells we found no effect of genistein on the adenylate cyclase activity. Nevertheless, it is clear from our data that phosphorylation status of CFTR affected the kinetic parameters for activation of wild type CFTR by genistein: with phosphorylated CFTR, the activation is greatly reduced as compared to non-phosphorylated CFTR.

We also found that the kinetic parameters of activation by genistein are similar in wild type and G551D CFTR cells. This suggests that the NBD1 mutation does not interfere with the putative binding site for genistein on CFTR. If one genistein binding site is located within the NBD2 but not the NBD1 domain, as suggested by recent studies (Randak et al., 1999; Howell et al., 2000), then the occupancy of this site may depend on the phosphorylation status of the R-domain. This is critical for the gating mechanism of the CFTR chloride channel, which may be driven into a refractory state or, alternatively, to a highly activated state. In conclusion, a study of the actions of CFTR modulators may be useful in clarifying how the different domains of CFTR interact with each other to form a protein kinase A- and ATP-regulated Cl^- channel and how mutations in CFTR cause cystic fibrosis.

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