Tyrosine Kinase–Independent Extracellular Action of Genistein on the CFTR Cl⁻ Channel in Guinea Pig Ventricular Myocytes and CFTR-Transfected Mouse Fibroblasts

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Abstract: The effects of genistein, a protein tyrosine kinase inhibitor, on the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel were studied in guinea pig ventricular myocytes and in NIH3T3 mouse fibroblasts stably transfected with CFTR cDNA by the whole-cell patch-clamp technique. Genistein did not activate whole-cell Cl⁻ currents when applied to the intracellular (pipette) solution. In contrast, when applied to the extracellular solution, genistein alone promptly activated the Cl⁻ current in a fully reversible manner. Also, extracellular genistein reversibly potentiated the forskolin-activated Cl⁻ current. However, both basal and forskolin-activated Cl⁻ currents were not affected by other protein tyrosine kinase inhibitors, including herbimycin A, lavendustin A, tyrphostin 21, tyrphostin 47, and tyrphostin 51. A nonspecific inhibitor of protein phosphatases, orthovanadate, had no effect on the genistein-induced activation of CFTR. Pretreatment with a protein kinase inhibitor, either H-89 or H-7, or with an adenylate cyclase inhibitor, SQ 22536, also had no effect on the genistein-induced response. Thus, it is concluded that genistein alone activates CFTR by a protein tyrosine kinase–independent and protein phosphatase–independent mechanism from the extracellular side, but not from the intracellular side. [Japanese Journal of Physiology, 48, 389–396, 1998]

Key words: genistein, CFTR, chloride channel, protein tyrosine kinase, protein phosphatase.

An isoflavonoid, genistein, which is known to be a potent inhibitor of protein tyrosine kinase (PTK) [1], has been shown to activate the epithelial cystic fibrosis transmembrane conductance regulator (eCFTR) Cl⁻ channel not only in heterologous eCFTR expression systems, including mouse NIH3T3 [2–7], rat IEC-6 [3], insect Hi-5 cells [7, 8], and Xenopus oocytes [9], but also in cells that express endogenous eCFTR, such as shark rectal glands [10], human T84 [3, 11], HT-29/B6 [11], and Calu-3 cells [6, 12].

The mechanism by which genistein stimulates CFTR is not precisely known. The earliest study [2] suggested that genistein may exert an activating action via inhibiting PTK-mediated phosphorylation of eCFTR or its regulator protein, whereas later studies [4, 8, 11] reported that the action of genistein may be best explained by a suppressing effect on some serine/threonine protein phosphatase that is involved in the deactivation (dephosphorylation) of eCFTR. However, several recent studies [5, 7, 9] provided evidence suggesting that the effects of genistein are caused by a direct action on eCFTR via a PTK-independent and protein phosphatase–independent mechanism.

Genistein was also demonstrated to activate the cardiac isofrom of CFTR (cCFTR), which is an exon 5 splice variant of eCFTR [13, 14], in guinea pig ventricular myocytes [15] and to potentiate forskolin- or isoproterenol-activated eCFTR Cl⁻ conductance in ventricular myocytes of the guinea pig [16–18] and rat hearts [19]. The inhibition of PTK has been suggested...
as a causal factor for the genistein actions on cCFTR [15–18], but other possible mechanisms have not been investigated. Thus the present study was first performed to answer the question of whether a PTK inhibitor, genistein, alone exerts an activating action on cCFTR in a PTK-dependent or -independent manner.

Since genistein is known to interact with the nucleotide binding sites of PTK [1], it has been thought that the action of genistein may be mediated by binding to the nucleotide binding domain of cCFTR from the intracellular site [5, 7, 9]. Thus the present study was also performed to answer this question: Is the action of genistein mediated by interaction with cCFTR and cCFTR from the intracellular or the extracellular side?

METHODS

Cell preparation. Ventricular myocytes were isolated from guinea pigs under pentobarbital anesthesia, as described previously [20]. They were suspended and stored at 4°C in KB solution containing (mM): L-glutamic acid 70, KCl 25, taurine 20, KH₂PO₄ 10, MgCl₂ 3, EGTA 0.5, glucose 10, and HEPES 10 (pH 7.35 adjusted with KOH).

NIH3T3 cells transfected with wild-type CFTR (NIH3T3/CFTR cells) [21] were provided by M. J. Welsh (University of Iowa) and cultured in monolayer in Dulbecco’s modified Eagle’s medium supplemented with streptomycin (40 μg/ml), penicillin (10 μg/ml), NaHCO₃ (10 mM), and 10% fetal calf serum. Suspensions of isolated NIH3T3/CFTR cells were prepared by detaching from the plastic substrate and culturing at 37°C with stirring for 10 to 60 min before experiments.

Electrophysiology. Aliquots of cell suspension were added to a perfusing chamber (1 ml) on the stage of an inverted microscope (TMD, Nikon, Tokyo) and perfused with a bathing solution at a rate of ~3 ml/min. Whole-cell recordings were performed at room temperature (23–25°C), as reported previously [22], by using a patch-clamp amplifier (Axopatch 200A; Axon Instruments, Foster City, CA). Ramp voltage pulses (0 to ±100 mV, every 8 or 10 s) were applied from a holding potential of 0 mV. Current signals were filtered at 1 kHz and digitized at 4 kHz.

The control bathing solution contained (mM): NaCl 103 (or 150), Na-glucuronate 47 (or 0), MgCl₂ 0.5, CdCl₂ 1, glucose 5.5, and HEPES 5 (pH 7.4 with NaOH). In some experiments, the concentrations of NaCl and Na-glucuronate were changed to 18 and 132 mM, respectively. The control pipette solution contained (mM) CsCl 85, EGTA 10, TEA-Cl 20, MgATP 10, Na₂-creatine phosphate 5, MgCl₂ 0.5, glucose 5.5, and HEPES 10 (pH 7.35 with CsOH). In some experiments, CsCl was replaced with Cs-aspartate. K⁺ currents were eliminated by internal TEA and omission of internal and external K⁺, and voltage-gated Na⁺ and Ca²⁺ currents were eliminated by inactivation at a holding potential of 0 mV. Any residual Ca²⁺ currents were removed by external Cd²⁺. Na⁺-K⁺ pump currents were eliminated by the omission of external K⁺, and Na⁺-Ca²⁺ exchanger currents were eliminated by the nominal absence of internal and external Ca²⁺.

Chemicals. All the agents except N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89; Calbiochem-Novabiochem Co.) and 1-[5-isoquinolinesulfonyl]-2-methylpiperazine dihydrochloride (H-7; Funakoshi Co.) were purchased from the Sigma Chemical Co. and added to the bath solution. Stock solutions of genistein, daidzein, tyrophostin 51, tyrophostin 47, tyrophostin 21, herbimycin A, lavedustin A, H-89, H-7, and 9-(tetrahydro-5-methyl-2-furyl) adenine (SQ 22536) were prepared by dissolving them in DMSO. Forskolin was dissolved in ethanol, and isoproterenol (ISO) and DIDS were dissolved in distilled water for stock solutions. In some experiments, genistein was added to the pipette solution or applied by the internal perfusion technique of Soejima and Noma [23] with slight modification.

Statistical analysis. All the data were given as mean ± SD. The statistical differences of the data were evaluated by Student’s t-test and were considered significant at p < 0.05.

RESULTS

Genistein activates CFTR Cl⁻ currents

In NIH3T3/CFTR cells, the extracellular application of 50 μM genistein reversibly activated whole-cell eCFTR Cl⁻ currents that exhibited virtually a linear current-voltage (I-V) relation (Fig. 1). The mean peak amplitudes of genistein-induced responses were 107.2±56.0 nA/nF at +100 mV and −109.7±68.1 nA/nF at −100 mV (n = 4). Extracellular genistein promptly potentiated forskolin-activated ohmic whole-cell eCFTR currents (Fig. 1). These results are in good agreement with previous single-channel studies in NIH3T3/CFTR [2, 5–7].

Current activation by the extracellular application of genistein was also found in guinea pig ventricular myocytes at 50 μM, as shown in Fig. 2 (A, B), but not at 10 μM (data not shown, n = 9). The genistein effect reversed rapidly after washout. Under asymmetrical Cl⁻ conditions (153 mM/21 mM), the reversal poten-
Fig. 1. Activation and potentiation of CFTR Cl− currents by genistein in NIH3T3/CFTR cells. The whole-cell Cl− current was recorded during a repetitive application of ramp pulses from 0 to ±100 mV under symmetrical Cl− conditions (106 mM). Upper: A representative chart record of whole-cell current before and after an extracellular application of genistein (indicated by horizontal lines). Lower: I-V curves from data in the upper panel (b, c, d) after subtraction of the basal current component (a).

The extracellular Cl− concentration ([Cl−]o) was reduced to 21 mM, from 153 mM, the reversal potential shifted to 0.4±2.3 mV (n=4), indicating preferential activation of Cl−-selective conductance. In the presence of 50 μM genistein, the I-V curve was nearly linear under the symmetrical Cl− conditions (Fig. 2A: lower panel). In the presence of 100 μM genistein, the mean amplitudes of peak current densities were 9.5±2.8 nA/nF at +100 mV and −6.0±1.7 nA/nF at −100 mV (n=8), exhibiting fairly outward rectification, under symmetrical Cl− conditions (106 mM: Fig. 2B). The outward rectification may be due to a blocking effect of genistein on the inward current at a high concentration (see below). As shown in Fig. 2B, the genistein-activated Cl− current was not blocked by extracellular application of 100 μM DIDS, which is well known to be ineffective against CFTR Cl− currents, including cAMP-activated Cl− currents in guinea pig ventricular cells [22]. These results show that genistein alone activates CFTR Cl− currents in guinea pig ventricular myocytes at concentrations higher than 50 μM, as previously reported [15].

At a lower concentration (10 μM), genistein potentiated forskolin-induced Cl− currents (Fig. 2C). The I-V curves were linear both before and after the application of genistein (Fig. 2C: lower panel). The mean peak values of forskolin-activated currents before and after the application of 10 μM genistein were 5.9±2.0 and 6.3±1.9 nA/nF, respectively, at +100 mV, and also −5.6±1.9 and −6.2±2.4 nA/nF (n=4), respectively, at −100 mV. These results are in good agreement with previous whole-cell studies in cardiac myocytes [16–18]. A higher concentration (50 μM) of genistein increased the outward current but suppressed the inward current, thereby changing the I-V relation from ohmic to outward rectification (Fig. 2C, lower panel). The mean peak currents in the presence of 50 μM genistein and 1 μM forskolin were 9.8±3.3 nA/nF at +100 mV and −5.9±1.9 nA/nF at −100 mV (n=8). An increase in the genistein concentration to 100 μM did not produce further effects (9.9±3.4 and −5.6±1.9 nA/nF at +100 and −100 mV, respectively: n=10). Thus it appears that genistein stimulates CFTR Cl− currents but blocks the inward (but not outward) component at concentrations higher than 50 μM. It is possible that there is a difference in the effective concentration for the blocking effect on the inward current between cCFTR and eCFTR, because genistein only slightly affected eCFTR currents in NIH3T3/CFTR cells at 50 μM (Fig. 1). In fact, a similar blocking effect was recently observed at higher concentrations of genistein (≥75 μM) on single-channel activities of eCFTR (at −50 mV) [7].

Genistein action is independent of protein tyrosine kinase and protein tyrosine phosphatase

A genistein analog, daidzein, which has a minimal effect on PTK [1], failed either to activate Cl− currents or to potentiate forskolin-activated Cl− currents in guinea pig ventricular myocytes (Fig. 3A: n=5). Based on identical observations, Shuba et al. [15], Chiang et al. [16], Shuba and McDonald [17] and Hool et al. [18] suggested that the genistein actions are mediated by an inhibition of PTK in guinea pig ventricular myocytes. However, this suggestion could not be supported by experiments with other PTK inhibitors, which are structurally different from genistein. As shown in Fig. 3 (B–D), herbimycin A, lavendustin A, and tyrphostin 51 (TPS 51) failed to induce activation of Cl− currents and potentiation of forskolin-activated Cl− currents in guinea pig ventricular myocytes (n=4 each). Similarly, tyrphostin 47 and tyrphostin 21 were not effective (data not shown, n=4 and 3).

To test whether the CFTR activity is negatively regulated by PTK-induced tyrosine phosphorylation and positively regulated by protein tyrosine phosphatase–induced dephosphorylation, the effect of orthovana-
Fig. 2. Activation and potentiation of CFTR Cl⁻ currents by genistein in guinea pig ventricular myocytes. The whole-cell Cl⁻ current was recorded during a repetitive application of ramp pulses from 0 to ±100 mV. A: Effects of changes in the extracellular Cl⁻ concentration ([Cl⁻]ₑ) on the current activated by genistein applied to the extracellular solution. Upper: A representative chart record of whole-cell current under asymmetrical (153 mM/21 mM) and symmetrical (21 mM) Cl⁻ conditions. Lower: I-V curves from data in the upper panel (b, c) after subtraction of the basal current component (a). B: Effects of DIDS on the genistein-activated current under symmetrical Cl⁻ conditions (106 mM). C: Effects of genistein on forskolin-activated current. Upper: A representative chart record of whole-cell current under symmetrical Cl⁻ conditions (106 mM). Lower: I-V curves from the data in upper panel (b, c, d) after subtraction of the basal current component (a).

Genistein action is independent of protein kinase A, protein kinase C, and protein phosphatases

It was suggested that genistein inhibits dephosphorylation of eCFTR by inhibiting some protein phosphatases [4, 8]. If that were so, an inhibitor of protein kinase A (PKA) or protein kinase C (PKC) should decrease CFTR Cl⁻ currents by shifting the phosphorylation/dephosphorylation equilibrium in the direction of a higher dephosphorylation, because the CFTR activity is regulated by PKA- and PKC-induced phosphorylation [25]. The effects of pretreatment with a PKA inhibitor, H-89 (10 μM), or with a PKC inhibitor, H-7 (100 μM), on the genistein-induced response were observed. As shown in Fig. 4B, ISO-induced CFTR activation in guinea pig ventricular my-
Genistein interacts with CFTR from the extracellular side, but not the intracellular side

When a high concentration of genistein (150 μM) was applied to the cytosol for 5 to 15 min by using the intracellular perfusion technique, no detectable change in the basal currents was observed in guinea pig ventricular myocytes (data not shown, n=4). Also, as shown in Fig. 5A, Cl⁻ current activation was not induced by the continued presence (for 8 to 35 min) of genistein (150 μM) in the pipette (intracellular) solution (n=7). Even in the presence of intracellular genistein, however, an extracellular application of genistein (50 μM) could induce activation of cCFTR (Fig. 5A) with essentially identical current amplitudes (7.7±2.8 nA/nF at +100 mV, n=8).

Neither was any effect of intracellular genistein (50 μM) incorporated in the pipette solution observed in NIH3T3/CFTR cells, as shown in Fig. 5B (n=3). Moreover, similar to the cCFTR current, intracellular genistein did not affect the activation of cCFTR current induced by extracellular genistein. These results indicate that genistein exerts an activating action by
Fig. 5. Effects of intracellular genistein incorporated in the pipette solution on the basal current and the current activated by extracellular application of genistein. The whole-cell Cl⁻ current was recorded during a repetitive application of ramp pulses from 0 to ±100 mV under symmetrical Cl⁻ conditions (106 mM). A: In a guinea pig ventricular myocyte. B: In an NIH3T3/CFTR cell.

interacting with CFTR from the extracellular side, but not from the intracellular side.

DISCUSSION

A PTK inhibitor, genistein, activates both the epithelial and cardiac isoforms of CFTR. Since CFTR is a function of protein kinase and phosphatase activities [25, 27], some protein kinases and phosphatases, besides the CFTR protein per se, may provide the target of genistein. Therefore at least four possible mechanisms may account for the effects of genistein on CFTR: 1) Via the inhibition of PTK, which is involved in phosphorylation of CFTR tyrosine residue, thereby down-regulating the CFTR Cl⁻ channel; 2) Via the activation of PKA and PKC; 3) Via the inhibition of some protein phosphatases that are involved in the deactivation (dephosphorylation) of CFTR; and 4) Via the direct action on CFTR. Actually, the first [2], third [4, 8, 11], and fourth [5, 7, 9] possibilities were previously suggested, whereas only the first possibility was reported for cCFTR [15–18].

The absence of any effect of daidzein (Fig. 3A) may support the first possibility. However, a variety of other PTK inhibitors (lavendustin A, herbmecin A, tyrphostin 21, tyrphostin 49, and tyrphostin 51) failed to mimic the effects of genistein (Fig. 3B–D). Furthermore, because the genistein action was not abolished by orthovanadate, which is known to inhibit protein tyrosine phosphatase [24], the first possibility should be excluded (Fig. 4A). Neither has any inhibitory effect of orthovanadate been observed in NIH3T3/CFTR [7] and CFTR-transfected Xenopus oocytes [9]. In fact, no evidence is found for the PTK-induced tyrosine phosphorylation of CFTR after stimulation with forskolin and IBMX in COS-7 cells transfected with CFTR [28].

Many studies have provided evidence against the possibility that genistein somehow activates PKA. Neither an increase in the intracellular cAMP [2, 10] nor an activation of PKA [4] was observed under CFTR stimulation with genistein. CFTR activation by genistein was not inhibited by a specific inhibitor of PKA, PKI [7, 9]. In the present study, an adenylate cyclase inhibitor, SQ 22536, did not affect the genistein-induced activation of CFTR. Furthermore, not only did a PKA inhibitor, H-89, fail to inhibit the genistein-induced activation of CFTR, but so did a PKC inhibitor, H-7 (Fig. 4, B, C). These results clearly reject the second possibility.

The lack of effect of a PKA inhibitor on the genistein action may also exclude the third possibility, because the level of CFTR activity is determined by the equilibrium between PKA-induced phosphorylation and protein phosphatase-mediated dephosphorylation of the CFTR protein. Also, a recent study [5] has provided evidence against the third possibility by observing that phosphatase-resistant thiophosphorylation could prime the CFTR Cl⁻ channel for genistein-induced activation.

Thus only the fourth possibility remains. The prompt activation of CFTR Cl⁻ currents on genistein application and rapid deactivation after the washout of genistein may support a direct interaction between the drug and the CFTR protein. Recently, direct interaction via binding to the nucleotide binding site of CFTR was suggested [5, 7, 9] because genistein is known to bind to the nucleotide binding sites of PTK [1]. However, it appears that genistein interacts with the extracellular domain of CFTR because the present study demonstrates, for the first time, that the drug action was observed in guinea pig ventricular myocytes and also in NIH3T3/CFTR cells under a whole-cell clamp, only when applied to the bulk extracellular (bath) solution but not to the intracellular (pipette) solution (Fig. 5). Previous inside-out patch-clamp studies showed that genistein is effective when applied to the cytosolic side [5, 7–9]. However, this “intracellular” effect may have been apparently induced by extracellular genistein after permeating through the patch membrane to the extracellular (pipette) solution, the volume of which is much smaller than that of the bulk intracellular (bath) solution in the inside-out mode.

Taken together, it is concluded that genistein alone activates epithelial and cardiac CFTR Cl⁻ channels by
interacting with CFTR from the extracellular side in a protein tyrosine kinase-independent and protein phosphatase-independent manner. The exact mechanism of the extracellular action of genistein remains for further investigations.

CFTR is a cAMP-activated Cl⁻ channel, which plays an essential role in epithelial membrane Cl⁻ transport [25] and in the modulation of cardiac action potentials [20, 27, 29]. Mutations of the single gene encoding CFTR cause cystic fibrosis, the most common lethal genetic disease in Caucasians [30]. Recently a PTK inhibitor, genistein, was found to activate not only the wild-type CFTR Cl⁻ channel, but also the most common cystic fibrosis-associated mutant CFTR (ΔF508-CFTR) [6]. Therefore genistein (or some ligand that can mimic the genistein action, if in fact there is any) may be of therapeutic benefit in the treatment of cystic fibrosis.

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