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Potentiation of mutant CFTR Cl^- channel currents by the naturally occurring stilbene compound resveratrol

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Previously, we found that the naturally occurring stilbene compound resveratrol (RES) could potentiate cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel activity. Because some wild-type CFTR activators also potentiate its mutant forms, we investigated effect of RES on the two most common forms of CF-related mutation (ΔF508 and G551D-CFTR). Cell-based fluorescence studies indicated that RES dose-dependently potentiated both ΔF508 and G551D mutant CFTR Cl^- channel activities. Transepithelial Cl^- currents were stimulated by RES in ΔF508 and G551D mutant CFTR-expressing FRT cells. Further excised inside-out patch-clamp measurements revealed that RES significantly induced the chloride current of ΔF508 and G551D mutant CFTRs by increasing the open time of the channels. In *ex vivo* studies, RES stimulated fluid secretion in mouse trachea by optical measurement of single gland secretion. These data suggested that RES is a potent ΔF508 and G551D mutant CFTR potentiator, and RES may present a novel class of therapeutic lead compounds in treating cystic fibrosis.

1. Introduction

Cystic fibrosis (CF, MIM#219700) is the most common leathal genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes for CFTR. CFTR is a cAMP-dependent chloride channel that belongs to ATP-binding cassette (ABC) transporter family (Riordan et al. 1989). CFTR is ubiquitously expressed in the apical membrane of all absorption and secretory epithelial cells, where it plays a crucial role in transepithelial fluid homeostasis (Kunzelmann 1999; Greger 2000; Banales et al. 2006; Riordan 2008).

The CFTR gene was identified in 1989 as the gene mutated in CF (Riordan et al. 1989; Kerem et al. 1989). So far, more than 1600 mutations of the CFTR gene have been identified and assigned to five classes according to the fate of their final products. ΔF508 and G551D mutant CFTRs are the most common CFTR mutations that lead to CF, which accounts for more than 75% of CF patients. ΔF508 mutant CFTR is extremely inefficient in that it leads to protein misfolding, retention by the endoplasmic reticulum, and degradation (Cheng et al. 1990; Dalemans et al. 1991). The small portion of mutated protein that escapes from the ubiquitous protease system still has defect in channel gating. G551D mutant CFTR is the 2nd most common mutation that leads to CF, which has also severe impaired channel gating defect (Cheng et al. 1990; Gregory et al. 1991).

Gating mutations associated with defective conductance can be modulated by CFTR potentiators. Identification of wild-type and mutant CFTR activators has been highlighted in previous drug discovery efforts (Ma et al. 2002; Yang et al. 2003; Xu et al. 2008). In a previous study, we found that resveratrol (3, 5, 4'-trihydroxystilbene, RES) stimulated CFTR-mediated Cl^- current in FRT cells expressing wild-type CFTR (wt-CFTR) and induced Cl^- current in isolated mouse colonic mucosa (Yang et al. 2013). Because many wt-CFTR potentiators also potentiate its mutant forms, we were curious to know whether RES potentiates ΔF508 and G551D mutant CFTR chloride channel activities. In this study, we again used cell-based fluorescence assays, transepithelial short-circuit current measurements and excised inside-out patch-clamp analysis. Airway liquid hyperabsorption is a key pathophysiological symptom and represents one of the leading causes of mortality in CF, so, we also investigated effect of RES on live tissue mouse trachea mucosa.

2. Investigations and results

2.1. Potentiation of ΔF508 and G551D mutant CFTR Cl^- channel activity in transfected FRT cells by RES

The effect of RES on ΔF508 and G551D mutant CFTR chloride channel activities was primarily evaluated using cell-based fluorescence assay. Because ΔF508-CFTR has both localization and channel gating defects, ΔF508-CFTR-expressing FRT cells were first incubated at 27∼30 °C for at least 16 h to allow sufficient CFTR protein rescued in the apical membrane (Pedemonte et al. 2005). Cells were then pre-stimulated with 20 μM forskolin (FSK). PBS and genistein were used as negative and positive control, respectively. These experiments showed that RES modestly but significantly activated both ΔF508 and G551D mutant CFTR-mediate iodide influx (Fig. 1A); EC_{50} values of the activations were all around 150 μM in both cases as the dose-response data shown in Fig. 1B.

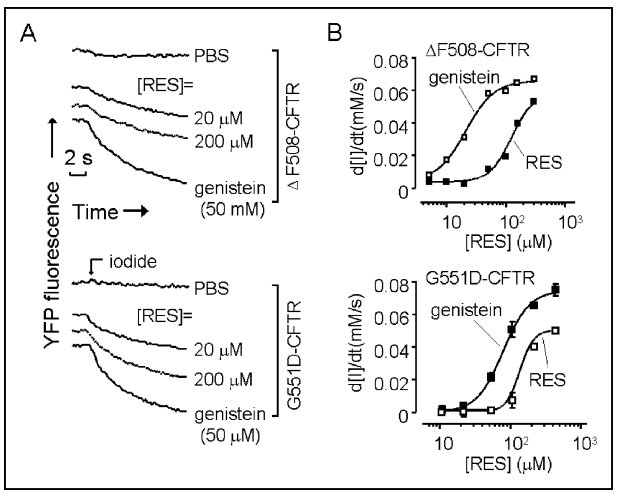

Fig. 1: Potentiation of ΔF508 and G551D mutant CFTR choloride channel activities by RES in CFTR-expressing FRT cells. (A) Typical time-course potentiation curves of ΔF508 and G551D mutant CFTR by indicated concentration of RES. Assays were performed in the presence of 20 μM FSK. PBS and genistein were used as negative and positive control, respectively. (B) Dose-response relationships showing the sensitivity of ΔF508-CFTR (top) and G551D-CFTR (bottom) to RES and genistein. PBS with same concentrations of DMSO was used as negative control. Cells were co-stimulated with 20 μM FSK and test compounds. Data are expressed as Mean ± SE, n = 6.

The activities were further confirmed by short-circuit current assays. RES induced Cl^- currents in a concentration-dependent manner, an increased Isc (ΔIsc) of $\sim 0.74 \pm 0.06\ \mu A/cm^2$ was induced by 100 μM RES in ΔF508-CFTR (Fig. 2A, 2C), and $5.5 \pm 0.6\ \mu A/cm^2$ of ΔIsc was induced by 20 μM RES in G551D-CFTR (Fig. 2B, 2D). $CFTR_{inh}$-172 completely inhibited the RES-induced Cl^- current, indicating the induced Cl^- current is CFTR-mediated. RES was also tested on ΔF508-CFTR misprocessing correction activities, no significant effect was found in the fluorescence assays (data not shown).

2.2. Excised inside-out patch clamp recordings of RES-stimulated CFTR activities

Effects of RES on ΔF508 and G551D mutant CFTR chloride channel activities were further confirmed by patch clamp studies, in which the channels were first activated by phosphorylation to a steady state with 25 U/mL PKA and 1 mM ATP, then with 1 mM ATP plus indicated concentration of RES. Figure 3A shows a continuous current trace from an excised inside-out patch of a FRT cells expressing ΔF508-CFTR. The results indicated that the increase of the ΔF508-CFTR channel activity by RES is achieved mainly by an increase in the open time of the channel. Similar results were obtained for G551D-CFTR expressed in FRT cells (Fig. 3B). Quantitative data are shown in Fig. 3C: ~370% increased of current was induced by 100 μM RES in G551D-CFTR, and ~120% increased of current was induced by 100 μM RES in ΔF508-CFTR. The activation is reversible, as removal of RES recovers all the currents (Fig. 3C).

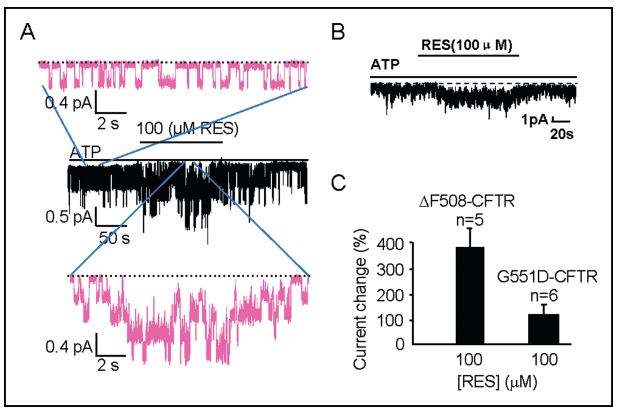

Fig. 3: Effects of RES on ΔF508 and G551D mutant CFTR activity in excised inside-out patch clamp recordings. (A, B) Representative traces of ΔF508 (A), and G551D (B) mutant CFTR from an inside-out patch showing a reversible potentiation of the channel activity by RES. In an inside-out patch clamp experiment, after the channels in the patch were activated with 1 mM MgATP plus PKA, 1 mM MgATP, 1 mM MgATP plus indicated concentration of RES were applied sequentially. (C) Averaged current changes from indicated number of similar recordings as in normalized to basic current (1 mM MgATP control group).

2.3. Potentiation of mutant CFTR chloride channel activities in live tissue by RES

CFTR is primarily expressed at the apical surface of airway epithelial cells and plays an essential role in maintaining mucociliary clearance by regulating the airway surface liquid. Effect of RES on fluid secretion was visualized in excised fragments of cranial portion of mouse trachea under low-magnification light microscopy. Gland fluid secretion was stimulated by addition of 100 μM pilocarpin or 100 μM RES to the serosal bathing solution. Figure 4A shows a series of images of expanding fluid droplets secreted by single submucosal glands after stimulation by RES with pilocarpine as positive control. Figure 4B summarizes the fluid secretion rate of individual submucosal gland stimulated by the indicated maneuvers. RES stimulated fluid secretion that was inhibitable by $CFTR_{inh}$-172.

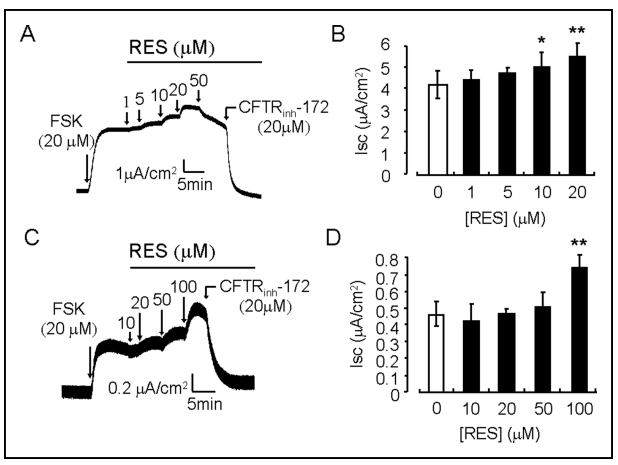

Fig. 2: Effects of RES on ΔF508 and G551D mutant CFTR chloride channel currents. (A, C) Short-circuit current measurement of activation of mutant CFTR by RES. Representative Ussing chamber traces showing ΔF508-CFTR (A) and G551D-CFTR (C) currents upon stimulation with FSK (20 μM) and the indicated concentrations of RES. Graphic representation of RES stimulation of transepithelial Cl − conductance in FRT cells expressing ΔF508 (B) and G551D (D) CFTR. Data are expressed as Mean ± SE, n≥6, $^*p < 0.05$, $^{**}p < 0.01$.

2.4. Charecteristics of RES potentiation of ΔF508 and G551D -CFTR Cl^- channel activity in transfected FRT cells

To investigate the mechanism of ΔF508 and G551D mutant CFTR activation by RES, we tested FSK-dependent properties of the activation. Cell-based fluorescence analysis indicated that activation of ΔF508 and G551D mutant CFTR by 200 μM RES depends on CFTR phosphorylation level (Fig. 5A, 5B).

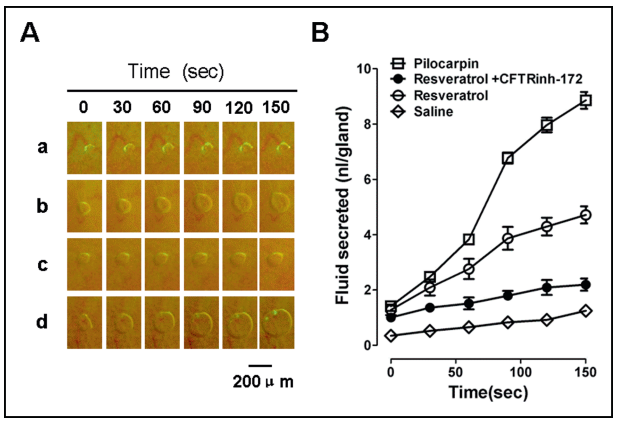

Fig. 4: RES stimulates fluid secretion in submucosal glands of mouse trachea. (A) Bright field micrographs of expanding fluid droplets secreted from single submucosal glands. a, saline; b, RES; c, RES plus CFTRinh-172; d, pilocarpin. (B) Linear volume increase of gland droplets (SE, n = 6) with time after stimulation.

3. Discussion

In the present study, we showed that RES is a potentiator of ΔF508 and G551D mutant CFTR chloride channels. Potentiation effects of RES on mutant CFTR chloride channel activities were primarily tested using a cell-based fluorescence method (Galietta et al. 2001), showing that RES stimulated both mutant CFTR-mediated iodide influxes in a FSK-dependent manner. Short-circuit current analysis indicated that RES induced significant increase of Cl^- current in the presence of 20 μM FSK. The potentiation effects of RES on ΔF508 and G551D mutant CFTR chloride channel activities were also confirmed by excised inside-out patch clamp studies. Further, we investigated the CFTR chloride channel potentiation ability of RES on live tissue. In the airway submucosal gland stimulation test, 100 μmol/L RES stimulated ∼4-fold more rapid fluid secretion *vs* saline control. The stimulated secretions could be fully abolished by $CFTR_{inh}$-172.

RES is a naturally occurring stilbene compound, produced by a wide variety of plants including grapes, berries, and peanuts (Nakata et al. 2012). RES was first isolated from the roots of the white hellebore (*Veratrum grandiflorum* O. Loes) in 1940 (Takaoka 1940), then from the roots of *Polygonum cuspidatum* in 1963 (Nonomura et al. 1963). RES has been receiving worldwide attention because of its variety of health benefits and numerous favorable biological activities in cancers, cardiovascular diseases, as well as ischemic injuries (Jang et al. 1997; Bradamante et al. 2004; Wang et al. 2002). Though beneficial effects of RES have been fully confirmed, literature on RES biological activities so far is contradictory and confusing

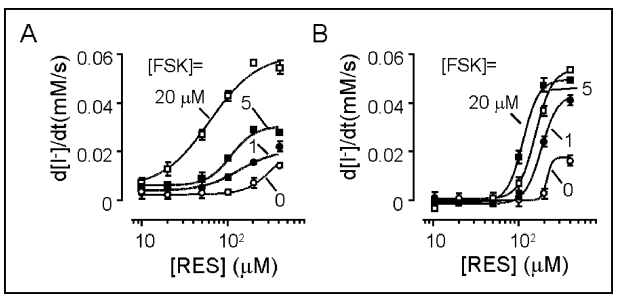

Fig. 5: FSK-dependent properties of ΔF508 and G551D mutant CFTR potentiation by RES in transfected FRT cells. (A) Dose-response relationships of ΔF508-CFTR potentiation by RES at indicated concentrations of FSK. (B) Dose-response relationships of G551D-CFTR potentiation by RES at indicated concentrations of FSK. Data were expressed as Mean ± SE of 6 independent tests.

(Nakata et al. 2012). The wide range of concentrations used in different studies may account in part of the discrepancy. In addition, previous studies indicated that RES exhibited low oral bioavailability and underwent rapid first-pass metabolism (Kapetanovic et al. 2010), this raises the questions about the concentrations achievable *in vivo*. Because a large number of cellular effectors have been proposed as potential targets for RES actions (Kutuk and Basaga 2007; Tyagi et al. 2005; Surh et al. 2001; Ulrich et al. 2005), a synergetic effect of RES on different molecular targets may provide an explanation for the biological activities of RES. In the present study, we identified CFTR as a new molecular target of RES, which may provide a new clue to understand the molecular basis for effects of RES.

CF affects many organs, pulmonary manifestations account for over 90% of the morbidity and mortality (Pier 2012). Normally, CFTR is expressed in ciliated airway epithelial cells of the surface and submucosal gland ductal epithelium (Toczyłowska-Mamińska and Dołowy 2012), and in the fluid-secreting cells of the submucosal glands (Ballard and Spadafora 2007). It has been well documented that CFTR function contributes to several airway diseases like CF and bronchiectasis (Boucher et al. 2010; Bienvenu et al. 2010). Restoring activities of impaired CFTR Cl^- channels might be a potential treatment of CFTR related diseases. Here, we found that RES could potentiate ΔF508 and G551D mutant CFTR Cl^- channels, which suggests a potential use of RES in the treatment of CF. Moreover, our results on mouse trachea mucosa, rat colonic mucosa (Yang et al. 2013), as well as other's (Blumenstein et al. 2005; Alexander et al. 2011) demonstrated that RES is effective on native cell systems expressing endogenous CFTR, which highlights its possible clinic use in CFTR-related diseases including respiratory diseases.

In cells transfected with G551D-CFTR and ΔF508-CFTR, the basal currents were much lower due to impaired channel gating activities of these mutants. Application of RES (100 μM) induced a current with an amplitude of 4.7 and 2.2 fold of the basal level in FRT cells transfected with G551D-CFTR and ΔF508-CFTR, respectively. These results demonstrated that RES can directly activate CFTR mutant channels in the absence of cAMP and PKA signaling pathway, which correlates well with our former results (Yang et al. 2013).

In conclusion, the present study identified a new activity of RES in potentiating ΔF508 and G551D mutant CFTR Cl^- channels. RES may present a novel class of therapeutic lead compounds in treating cystic fibrosis.

4. Experimental

4.1. Chemicals

RES was obtained from the National Institute for the Control of Pharmaceutical and Biological Products in China. $CFTR_{inh}$-172 was synthesized as refference (He et al. 2004). Forskolin (FSK) and genistein were purchased from Sigma Chemical Co. (St. Louis, MO). Test compounds were dissolved in DMSO to make mother solution (20 mM) and stored at -80 °C. Concentration of DMSO was less than 1% to ensure produce no significant effect on tests.

4.2. Cell culture

Fischer rat thyroid epithelial (FRT) cells co-expressing YFP-H148Q/I152L and ΔF508 (or G551D) mutant CFTR were made as reference (Galietta et al. 2001; Zegarra-Moran et al. 2002). The FRT cells were maintained in F-12 Coon's medium (Sigma Chemical Co. St. Louis, MO. U.S.A.) supplemented with 10% characterized fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a CO_2 incubator, at 37 °C.

4.3. Iodide influx fluorescence assay

The FRT cells were seeded into a black-walled, clear-bottomed 96-well tissue culture plate (Costar, Corning, NY, USA) at high density (about

30,000 cells per well), and cultured for ~24 h in CO_2 incubator. After being washed three times with PBS (in mM: 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$), the cells were incubated with 20 μM FSK for 5 min and 20 μM FSK plus different concentrations of test compounds for another 10 min. Changes of the YFP fluorescence data were recorded using a microplate reader (Fluostar Optima, BMG Lab Technologies). Excitation and emission wavelength were set at 500 ± 10 nm and 535 ± 15 nm, respectively, by using specific filters (Chroma Technology Corp., Brattleboro, VT). Iodide influx rates (d[I^-]/dt) were computed as reported methods. Details were described in reference (Ma et al. 2002; Yang et al. 2003).

4.4. Ussing chamber recordings

In Ussing chamber studies, the FRT cells were cultured into Snapwell permeable supports (Corning Life Sciences). After the cells were confluent (~24 h), the cells were incubated at air-fluid interface for ~7 days to let tight junction formation. The basolateral membrane of the FRT cells was permeabilized with 250 μg/ml amphotericin B. Transepithelial Cl^- currents were recorded using a vertical diffusion chamber (Physiological Instruments, San Diego, CA, USA) under an asymmetric transepithelial Cl^- concentration. All measurements were performed at 37 °C, and solutions were continuously bubbled with air. Transepithelial Cl^- currents were recorded with a DVC-1000 voltage clamp (World Precision Instruments, Sarasota, FL, USA). Details were described by Kopeikin et al. (2010).

The basolateral side solution contained (in mM): 130 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, 10 Na-HEPES, 10 glucose, pH 7.3; The apical side solution contained the same components except that 56 mM NaCl was replaced by 56 mM sodium gluconate and the concentration of $CaCl_2$ was increased to 2 mM.

4.5. Submucosal gland fluid secretion stimulation determination method

Effect of RES on mouse trachea submucosal fluid secretion was determined as reference (Song et al. 2009). Briefly, freshly excised mouse trachea was immediately mounted on a perfusion chamber with the mucosal side up and soaked in KH buffer (contained in mM: 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 24.8 $NaHCO_3$, 2.5 $CaCl_2$, 11.1 glucose, pH 7.4). After the mucosa was cleared with saline and dried by nitrogen stream, 40 μl saline saturated mineral oil was added to the surface. Gland fluid droplets were imaged and recorded by a reflected light microscopy (Olympus, Olympus Micro DP Controller) after stimulation with compounds. In some experiments, the tissues were incubated in KH-buffered $CFTR_{inh}$-172 (20 μM) for 30 min (in a CO_2 incubator, at 37 °C) before mounted on the perfusion chamber. The rate of fluid secretion from individual submucosal gland was computed from fluid droplet diameter assuming semi-spherical droplet geometry as the reference.

4.6. Patch-Clamp

The FRT cells were plated onto cover glasses for inside-out patch recordings. CFTR currents were recorded with an EPC10 amplifier (HEKA, Lamberecht/Pfalz, Germany) as reference (Kopeikin et al. 2010). In general, patch-clamp electrodes were made from B15024F glass capillaries, and fire polished to yield a resistance of 3–5 MΩ in bath solution. Membrane patches were excised into an inside-out mode after the seal resistance was > 40 GΩ. The pipette was perfused with protein I/O solution containing kinase A (PKA, 25 U/ml) and ATP (1 mM) until the CFTR current reached a steady state, and then exposed to RES in the ATP-containing I/O solution. CFTR currents were filtered at 100 Hz with an eight-pole Bessel filter (Warner Instrument) and captured at a sampling rate of 500 Hz. The membrane potential was held at − 50 mV in all experiments.

The standard bath solution contained (in mM): 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 20 sucrose and 5 HEPES, pH 7.4 with NaOH. The electrodes solution contained (in mM): 140 N-methyl-D-glucamine chlorides (NMDG-Cl), 2 $MgCl_2$, 5 $CaCl_2$, and 10 HEPES, pH 7.4 with NMDG. The I/O solution contained (in mM): 150 NMDG-Cl, 10 EGTA, 10 HEPES, 8 TRIS, 2 $MgCl_2$, pH 7.4 with NMDG.

4.7. Statistical Analysis

All data are expressed as mean ± SE or as representative traces. Student's *t* test was used to compare test and control values, *P* values less than 0.05 were considered statistically significant.

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