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CFTR chloride channel is a molecular target of the natural cancer preventive agent resveratrol

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The naturally occurring polyphenol compound resveratrol (RES) has been receiving wide attention because of its variety of health benefits and favourable biological activities. Previous studies have shown that RES could induce intestinal chloride secretion in mouse jejunum and stimulate cAMP-dependent Cl^- secretion in T84, primary cultured murine nasal septal and human sinonasal epithelial cells, but the precise molecular target is not clear. We therefore tested the hypothesis that RES may stimulate the activity of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. Using cell-based fluorescent assays, transepithelial short-circuit current measurements and excised inside-out patch-clamp analysis; we found that RES dose-dependently potentiate CFTR Cl^- channel activities, which was reversed by CFTR inhibitors $CFTR_{inh}$-172 and GlyH101. Transepithelial Cl^- secretion by CFTR-expressing FRT cells was stimulated by RES with half maximal concentration ~80 μM. Intracellular cAMP content was not elevated by RES in FRT cells. Excised inside-out patch-clamp analysis indicated that RES significantly increased the chloride currents of CFTR. In *ex vivo* studies, RES stimulated the transmucosal chloride current of rat colon by short-circuit current assay. These data suggested that CFTR is a molecular target of RES. Our findings add a new molecular target to RES, and RES may represent a novel class of therapeutic lead compounds in treating CFTR-related diseases including CF and habitual constipation.

1. Introduction

Resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring polyphenol compound, which is produced by a wide variety of plants, including red grapes, mulberries, and peanuts when response to injury, UV-irradiation, and/or pathogen infections (Fremont 2000). RES has been receiving wide attention because it has been found to have a variety of health benefits and favorable biological activities such as antioxidant, antimutagenic, anti-inflammatory, chemo-preventive, and anticancer activities in both cell and animal models (Baur and Sinclair 2006). RES is also regarded as the possible reason that a low incidence of cardiovascular diseases may coexist with intake of high fat diet - the phenomenon of the *French paradox* (de Lange et al. 2007). In 2005, (Blumenstein et al. (2005) demonstrated that RES stimulated cAMP-dependent Cl^- secretion in T84 cancer cells and anion secretion in mouse intestinal mucosa. Based on this, they proposed that cAMP stimulated Cl^- secretion activity is a possible reason for severe diarrhea triggered among patients who had required certain plant polyphenols. Recently, Alexander (2011) indicated that RES could potentiate transepithelial Cl^- transport in primary cultured murine nasal septal (MNSE) and human sinonasal epithelial (HSNE) cells. Though, so far, various 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), the precise molecular mechanism whereby RES-induced chloride secretion remained largely elusive.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ATP-binding cassette (ABC) transporter family (Riordan et al. 1989), which is ubiquitously expressed in apical membrane of serous epithelial cells in intestines, airways, pancreas, bile ducts, epididymis, and conjunctiva (Kunzelmann 1999; Greger 2000; Banales et al. 2006; Riordan 2008). CFTR plays a crucial role in transepithelial fluid homeostasis because it is the primary driver of fluid and water secretion. It has been fully confirmed that CFTR is the final common pathway for intestinal Cl^- secretion in response to various agonists (Ma et al. 2002). So, we hypothesize that RES may stimulate the activity of CFTR chloride channel.

In previous studies, in screening of 386 herbal compounds and 40,000 fractions prepared from 500 herbs most frequently used in traditional Chinese medicine, we found a large number of CFTR potentiators including RES. Purpose of the present study was to investigate systematically the effect of RES on CFTR Cl^- channels in cell culture models and freshly isolated rat colonic mucosa.

2. Investigations and results

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

Effect of RES on CFTR Cl^- channel gating was first test on FRT cells co-expressing CFTR and YFP. Iodide influx rates were

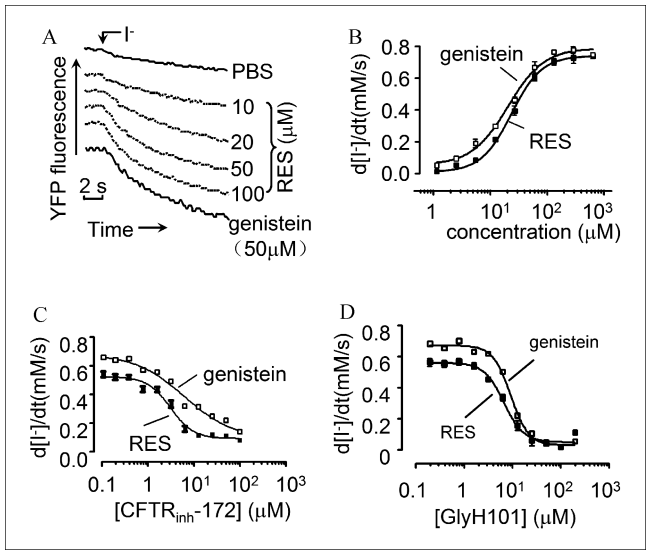

Fig. 1: RES potentiating wt-CFTR chloride channel activity in transfected FRT cells. (A) Typical time-course potentiation curves of wt-CFTR by indicated concentration of RES. Assays were performed in the presence of 0.1 μM FSK. PBS and genistein were used as negative and positive control, respectively. (B) Dose-response relationships showing similar sensitivity of wt-CFTR to RES and genistein. Data were expressed as Mean ± SE of 6 independent tests. (C-D) Potentiation of wt-CFTR chloride channel activity by 200 μM RES was inhibited by CFTR inhibitor $CFTR_{inh}$-172 (C) and GlyH101 (D). Data were expressed as Mean ± SE of 6 independent tests.

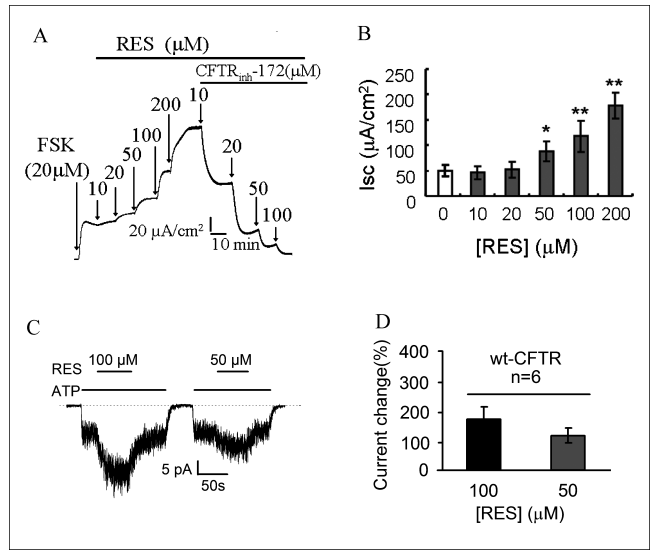

Fig. 2: Effects of RES on CFTR chloride channel currents. (A) Potentiation of short-circuit current (Isc) in permeabilized CFTR-expressing FRT cells by RES. Measurements were performed after the basolateral membrane of the FRT cells had been permeabilized with 250 mg/mL amphotericin B and in the presence of a 65 mM basolateral to apical transepithelial Cl^- gradient. Where indicated, various concentrations of RES were added to the apical chamber solutions. (B) Graphic representation of transepithelial Cl^- conductance stimulation in CFTR-expressing FRT cells. Data were expressed as Mean ± SE of 6 independent tests ($^*p < 0.05$, $^{**}p < 0.01$). (C) Representative traces of 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. (D) Average percentage current inc.

measured using a cell-based fluorescent assay as described in the Experimental Section. RES was tested at a series of concentrations and studies were done in the presence of 100 nM FSK. PBS (with same concentration of DMSO) and genistein (known CFTR potentiator) (Hwang et al. 1997) were used as negative and positive control, respectively. Original time-course traces are shown in Fig. 1A. RES, at 20 μM, stimulated significant increase of iodide influx. Dose-dependent relationship analysis indicated that RES and genistein showed similar efficiency and affinity to CFTR: EC_{50} and V_{max} values are ~25 μM and ~0.8 mM/S, respectively (Fig. 1B). Further fluorescence iodide influx analysis of FRT cells showed rapid CFTR Cl^- channel potentiation by 200 μM RES, with half-maximal activation time ($t_{1/2}$) less than 5 min. The effect is reversible, and the activity of wt-CFTR Cl^- channel restored to the basal level 15 min after removal of 200 μM RES (data no shown). In addition, potentiation of wt-CFTR by 200 μM RES could be inhibited by the known CFTR inhibitors $CFTR_{inh}$-172 and GlyH101 (Fig. 1C and 1D).

The activities were further confirmed by short-circuit current tests to ensure that the anion influx is CFTR-mediated. Tests were done in the presence of 65 mM basolateral to apical transepithelial Cl^- gradient and basolateral membrane of FRT cells permeabilized with 250 μg/ml amphotericin B. As shown in Fig. 2A and 2B, RES induced Cl^- current in CFTR-expressing FRT cells in a concentration-dependent manner. 200 μM RES induced a transepithelial short-circuit current (I_{sc}) as high as 180 μA/cm^2. The CFTR blocker, $CFTR_{inh}$-172, added at the end of tests completely blocked the current.

We also studied the effect of RES on CFTR Cl^- channels activities in excised inside-out membrane patches from FRT cells transfected with CFTR cDNA. The channels were first activated by phosphorylation to a steady state with 25 U/mL PKA and 1 mM MgATP, then with 1 mM ATP plus indicated concentrations of RES. Fig. 2C shows representative recordings of currents induced by RES. A 120% and 180% increase of currents were caused by 50 μM and 100 μM RES wt-CFTR, respectively, as summarized in Fig 2D. The RES-induced current was reversible, as removal of RES restored to the basal currents (Fig. 2C).

2.2. Characteristics of potentiation of CFTR Cl^- channel activity by RES in transfected FRT cells

CFTR activation can be achieved by (a) direct modulation of CFTR protein conformation and (b) activation of upstream signaling pathway (cAMP-dependent PKA pathway) (Hwang and Sheppard 1999; Sheppard and Welsh 1999; Schultz et al. 1999). To evaluate the relationship between CFTR phosphorylation and RES-induced activation, we studied RES activity under different FSK concentrations. As shown in Fig. 3A, RES is effective at inducing CFRT-mediated iodide influx without FSK, although its potency was proportionally lower. For example, the EC_{50} value was shifted from 60 μM (no FSK) to 20 μM (0.1 μM FSK). Previous studies showed that RES elevated cAMP concentration in T84 and MCF-7 cells (Surh et al. 2001; El-Mowafy and Alkhalaf 2003). We further evaluated the effect of RES on intracellular cAMP concentration in FRT cells. As shown in Fig. 3B, genistein caused a significant elevation of cAMP levels when combined with a low FSK concentration (0.1 μM) which is consistent with reported results (French et al. 1997). On the contrary, RES showed no significant effect on cAMP level in FRT cells either alone or with 0.1 μM FSK. High level of CFTR phosphorylation can be achieved by increasing intracellular cAMP-dependent phosphorylation (like FSK) and/or by inhibiting dephosphorylation of the protein (like IBMX) (Hwang and Sheppard 1999; Haws et al. 1996). Therefore we analyzed the synergetic effect of RES with FSK and IBMX. As shown in Fig. 3C, in the presence of high concentrations of FSK (20 μM) and IBMX (100 μM), RES still could elicit ~20% increase of iodide influx.

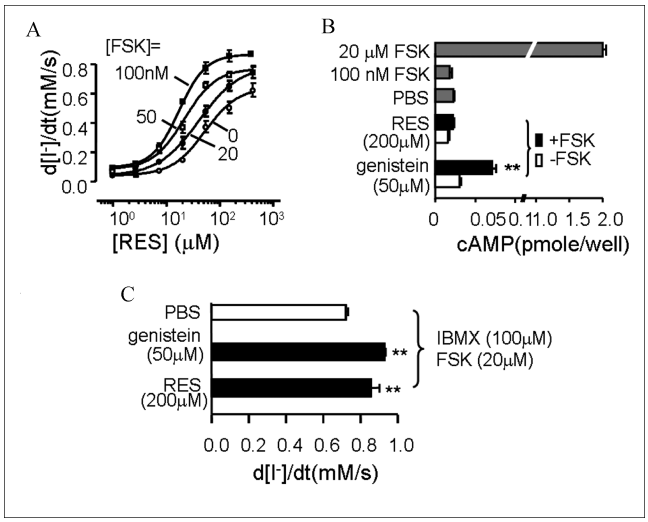

Fig. 3: Properties of wt-CFTR potentiation by RES in transfected FRT cells. (A) Dose-response relationships of wt-CFTR potentiation by RES at indicated concentrations of FSK. Data were expressed as Mean ± SE of 3 independent tests. (B) Intracellular cAMP levels of FRT cells measured under resting conditions, the FRT cells were incubated with FSK alone (0.1 or 20 μM), or with FSK (0.1 μM) plus 200 μM RES or with FSK (0.1 μM) plus 50 μM genistein. PBS with same concentration of DMSO was used as negative control. Genistein significantly increased cAMP levels, Data were expressed as Mean ± SE of 6 independent tests ($^{**}p < 0.01$). (C) Synergetic effect of RES with FSK and IBMX. Data were expressed as Mean ± SE of 6 independent tests ($^{**}p < 0.01$).

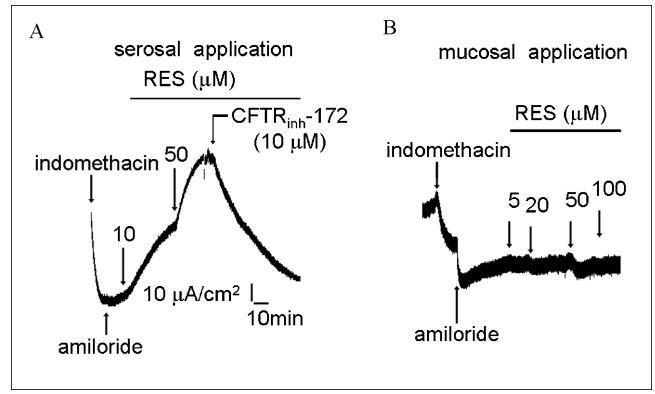

Fig. 4: Stimulation of Cl^- current in rat colonic mucosa by RES. RES-stimulated transepithelial Cl^- current was measured after inhibition of Na+ current by amiloride (10 μM) and prostaglandin production by indomethacin (10 μM). Where indicated, RES and CFTRinh-172 was added. RES was added to mucosal (A) and serosal (B) sides separately.

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

CFTR is expressed throughout the colonic epithelium and dominates in the crypts, so we used live tissue rat distal colonic mucosa to further confirm the CFTR-mediate Cl^- current potentiation activity of RES. The ability of RES to elicit transepithelial Cl^- currents in rat colonic mucosa epithelium was examined by recording short-circuits currents in Ussing chamber tests. RES was applied both mucosally and serosally to the mucosa. *I*sc values were continuously recorded in the presence of ENa inhibitor amiloride (10 μM) and prostaglandin generation inhibitor indomethacin (10 μM). It showed that only serosal application of RES (Fig. 4A) is effective. RES, at 50 μM, resulted in 100 $\mu A/cm^2$ current increases, while no significant currents were induced when RES was applied mucosaly (Fig. 4B) at a series of concentrations from 5 μM to 100 μM. $CFTR_{inh}$-172 completely abolished the chloride transport stimulated by serosaly applied RES.

3. Discussion

In the present study, we provided evidence that RES is a potentiator of CFTR chloride channels. Effect of RES on CFTR chloride channel activities was primarily investigated by using a cell-based fluorescence assay, in which FRT cells was stably cotransfected with CFTR and the halide sensitive YFP indicator (Galietta et al. 2001). By using FRT cells grown as monolayers, we were able to record CFTR-mediated iodide influx rates under different conditions. The fluorescence assay showed that RES stimulated CFTR-mediated iodide influx in a FSK-dependent manner; the activation could be abolished by the known CFTR inhibitors, $CFTR_{inh}$-172 and GlyH101. The same FRT cells were also used in short-circuit current analysis. Our data showed that RES at 200 μM significantly stimulated an increase of Cl^- current in the presence of low concentrations of FSK. The stimulatory effect of RES on CFTR chloride channel activity was also confirmed by excised inside-out patch clamp studies. Further, we investigated the CFTR chloride channel potentiation ability of RES on live tissues. Short-circuit current analysis was done on rat colonic mucosa in the presence of indomethacin and amiloride to inhibit prostaglandin formation and ENaC-mediate Na^+ currents. It showed that RES dose-dependently increased Cl^- current in the CFTR-transfected FRT cells, which suggested that effect of RES on CFTR does not involve in the enteric nervous system or the epithelial ENaC. These results clearly indicated that RES can effectively stimulate Cl^- transport and fluid secretion in live epithelial tissues and that the effect is through activation of CFTR chloride channel.

CFTR is closely related to many physiological and pathological situations. Apart from typical CF, dysfunction of CFTR in a single organ was associated with other CFTR-related disorders (CFTR-RD, clinical entities associated with CFTR dysfunction that does not fulfil the diagnostic criteria for CF) including habitual constipation (Pratha et al. 2000), congenital bilateral absence of the vas deferens (CBAVD, MIM#277180) (Yu et al. 2012), acute recurrent or chronic pancreatitis (Cohn et al. 1998), and disseminated bronchiectasis (Girodon et al. 1997). Restoring activities of impaired CFTR Cl^- channels might a potential treatment of CF as well as other CFTR-RDs. Our results on rat colonic mucosa as well as Alexander and cowokers' results on MNSE and HSNE cells demonstrated that RES is effective on native cell systems expressing endogenous CFTR, which suggests potential use of RES in the treatment of CFTR related symptoms such as habitual constipation. On the other hand, RES may cause severe diarrhea by enhancing intestinal secretory processes through activation of CFTR, a side effect that limits the potential use of RES as a chemopreventive agent (Blumenstein et al. 2005).

Activation of CFTR channels can be achieved by two ways. One way involves increasing cAMP levels and protein kinase PKA activity, which leads to increased phosphorylation at the R-domain of CFTR and thus activation of the channel. Another way is to activate the channel through direct interaction (Hwang and Sheppard 1999). Previous studies in T84 and MCF-7 cells found that RES could stimulate intracellular cAMP generation in those cells (Blumenstein et al. 2005; El-Mowafy and Alkhalaf 2003). In FRT cells, we did not find increased cAMP levels induced by RES although genistein did increase cAMP levels in our studies. RES was also reported by other investigators to have no effect on elevating cAMP levels in coronary artery smooth muscle cells (El Mowafy and White 1999; El-Mowafy et al. 2007). The effect of RES on activating cAMP-dependent pathways could be different depending on cell type of interest. Since RES does a not increase cAMP levels in FRT cells,

our studies suggest that RES activate CFTR channels in FRT cells by direct interaction with the channel. Patch clamp studies of RES-induced activation of CFTR channels further support the direction interaction mechanism. In inside-out patch clamp studies, PKA and MgATP was first used to activate the CFTR channels and induced a basal level current in wt-CFTR transfected cells. Subsequent application of 50 and 100 μM RES in the absence of PKA was able to induce a current with amplitude of 2.2 and 2.8 fold of the basal level, respectively. These studies demonstrated that RES can directly activate CFTR in the absence of cAMP and PKA signaling pathway.

In conclusion, these *in vitro* and *ex vivo* findings indicated that RES is a reliable wild-type CFTR-mediated Cl^- potentiator across different recording techniques. Our findings add a new molecular target to RES, and RES may represent a novel class of therapeutic lead compounds in treating CFTR-related diseases including CF, habitual constipation, and disseminated bronchiectasis.

4. Experimental

4.1. Chemicals

RES was purchased from the National Institute for the Control of Pharmaceutical and Biological Products in China. $CFTR_{inh}$-172 and GlyH101 were synthesized as described previously (He et al. 2004; Muanprasat et al. 2004). After several cycles of recrystalization, purity of compounds were >99.9% as confirmed by HPLC/MS analysis. Forskolin (FSK), genistein, amiloride, and indomethethin were purchased from Sigma Chemical Co. (St. Louis, MO). Compounds were dissolved as 20 mM stock solution in DMSO and stored at –80 °C. All compounds were diluted in PBS before experiments, and the final concentration of DMSO was <1% to ensure produce no significant effect on tests.

4.2. Cell culture

Fischer rat thyroid epithelial (FRT) cells were stably transfected with the YFP-H148Q/I152L fluorescence protein and CFTR cDNA as reference (Galietta et al. 2001; Zegarra-Moran et al. 2002). The cells were grown at 37 °C in F-12 Coon's medium (Sigma Chemical Co. St. Louis, MO. U.S.A.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a CO_2 incubator. The cells were used in iodide influx fluorescence study, short circuit current recording study and patch clamp experiments. In the iodide influx fluorescence study, the cells were seeded into a black-walled, clear-bottomed 96-well tissue culture plate (Costar, Corning, NY, USA) at a density of 30,000 per well; in short the circuit current recording study, the cells were seeded into Snapwell permeable supports (Corning Life Sciences) at 500,000 cells per insert.

4.3. Tissue preparation

All animal protocols were approved by the Ethics Committee of Jilin University. Sprague-Dawley (SD) rats were bred in the University specific pathogen free (SPF) animal centre. SD rats (body weight 200-220 g) were group-housed in a temperature-controlled room at 23 to 24 °C with a 12/12-hour light/dark cycle and allowed free access to food and water until the day of experiment. Animals were sacrificed by one over-dose of intravenous pentobarbital (100 mg/kg). The distal colon was immediately exteriorized and stripped of associated mesenteric and connective tissues, then placed in ice-cold Krebs-Henseleit (KH) solution (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. The segment of colon was opened along the mesenteric border, carefully dissected the mucosa. The isolated mucosal sheet was cut into an appropriate size and mounted in Ussing chambers (area 0.7 cm^2).

4.4. Iodide influx fluorescence assay

FRT cells were grown on 96-well plate to let confluent in ~24 h. The cells were 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 $MgCl_2$) and stimulated with FSK (FSK) for 5 min. Then different concentrations of test compounds were added to each well to a final volume of 40 μl and maintained for 10 min at 37 °C. YFP fluorescence was recorded by using a microplate reader (Fluostar Optima, BMG Lab Technologies) equipped with HQ500/20X (500 ± 10 nm) excitation and HQ535/30 M (535 ± 15 nm) emission filters (Chroma Technology Corp., Brattleboro, VT). Iodide influx fluorescence recording methods were similar for three kinds of CFTR, except for measurement of ΔF508-CFTR channel gating potentiation activity; the FRT cells were first rescued at 27 °C for 20 to 24 h prior to fluorescence quenching tests. Iodide influx rates ($d[I^-]/dt$) were computed from initial time-course fluorescence by third order polynomial regression method. Details were described in reference (Ma et al. 2002; Yang et al. 2003).

4.5. Ussing chamber recordings

Short-circuit current measurements were done ~7 days after the FRT cells were seeded in Snapwell inserts. The inserts were placed in a Ussing chamber system (Vertical Diffusion Chamber, Physiological Instruments, San Diego, CA, USA). Measurements were performed in an asymmetric transepithelial Cl^- concentration. 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, pH 7.3, and 10 glucose. The apical side solution contained the same components except that half NaCl was replaced by sodium gluconate and the concentration of $CaCl_2$ was increased to 2 mM to compensate for calcium buffering caused by gluconate. The basolateral membrane was permeabilized with 250 μg/ml amphotericin B.

For rat distal colonic mucosa studies, both hemisphere chambers contained (in mM) 126 NaCl, 0.38 KH_2PO_4, 2.1 K_2HPO_4, 1 $MgSO_4$, 1 $CaCl_2$, 24 $NaHCO_3$, and 10 glucose, basolateral membrane not permeabilized. Short-circuit current was measured after inhibition of Na^+ current by amiloride (10 μM) and prostaglandin generation inhibitor of indomethacin (10 μM), followed by stimulation by FSK (20 μM) and subsequent inhibitor addition (He et al. 2005).

Measurements were performed at 37 °C, and solutions were continuously bubbled with air. Short-circuit current was recorded with a DVC-1000 voltage clamp (World Precision Instruments, Sarasota, FL, USA) via Ag/AgCl electrodes and 1 M KCl agar bridges.

4.6. Patch-Clamp

Patch-clamp experiments were performed at room temperature (25 °C) with an EPC10 amplifier (HEKA, Lambrecht/Pfalz, Germany). FRT cells stably transfected with CFTR were plated onto cover glasses for inside-out patch recordings. Patch-clamp electrodes were made from B15024F glass capillaries (VitalSense Scientific Instrument). The pipette resistance was 3–5 MΩ in the bath solution. The membrane potential of the excised inside-out membrane patch was held at -50 mV for all experiments. Currents were filtered at 100 Hz with an eight-pole Bessel filter (Warner Instrument) and captured onto a hard disk at a sampling rate of 500 Hz. During experiments, FRT cells were first incubated in the bath solution containing (in mM) 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, and 5 HEPES (pH 7.4), and 20 mM sucrose was added to the bath solution to prevent activation of swelling-induced currents. The pipette 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. After giga ohm seal was formed, the membrane patch was excised into the I/O solution containing (in mM): 150 NMDG-Cl, 10 EGTA, 10 HEPES, 8 TRIS, 2 $MgCl_2$. CFTR current was activated by exposing the excised patch to the I/O solution containing protein kinase A (PKA, 25 U/ml) and ATP (1 mM). The control trace was obtained in the same I/O solution containing only ATP (1 mM) but not PKA. The patch was then exposed to RES in the ATP-containing I/O solution to evaluate the drug effect.

4.7. Intracellular cAMP content determination

Intracellular cAMP content was measured with a cAMP radio active immunoassay kit (Shanghai Traditional Chinese Medicine University). FRT cells grown on 96-well microplates were incubated with test compounds for 10 min without or with 0.5 μM FSK and then lysed. The lysates were assayed for cAMP content in sexplicate according to the manufacturer's instructions. Intracellular cAMP content was expressed as pmol per well.

4.8. Statistics

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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