Original Paper

Nobiletin Stimulates Chloride Secretion in Human Bronchial Epithelia via a cAMP/PKA-Dependent Pathway

Yuan Hao   Cindy S.T. Cheung   Wallace C.Y. Yip   Wing-hung Ko

School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong

Key Words

Nobiletin • Chloride secretion • cAMP/PKA • Bronchial epithelia • Flavonoid

Abstract

Background/Aims: Nobiletin, a citrus flavonoid isolated from tangerines, alters ion transport functions in intestinal epithelia, and has antagonistic effects on eosinophilic airway inflammation of asthmatic rats. The present study examined the effects of nobiletin on basal short-circuit current (I_{SC}) in a human bronchial epithelial cell line (16HBE14o-), and characterized the signal transduction pathways that allowed nobiletin to regulate electrolyte transport. ***Methods:*** The I_{SC} measurement technique was used for transepithelial electrical measurements. Intracellular calcium ($[Ca^{2+}]_i$) and cAMP were also quantified. ***Results:*** Nobiletin stimulated a concentration-dependent increase in I_{SC}, which was due to Cl^- secretion. The increase in I_{SC} was inhibited by a cystic fibrosis transmembrane conductance regulator inhibitor ($CFTR_{inh}$-172), but not by 4,4'-diisothiocyano-stilbene-2,2'-disulphonic acid (DIDS), Chromanol 293B, clotrimazole, or TRAM-34. Nobiletin-stimulated I_{SC} was also sensitive to a protein kinase A (PKA) inhibitor, H89, and an adenylate cyclase inhibitor, MDL-12330A. Nobiletin could not stimulate any increase in I_{SC} in a cystic fibrosis (CF) cell line, CFBE41o-, which lacked a functional CFTR. Nobiletin stimulated a real-time increase in cAMP, but not $[Ca^{2+}]_i$. ***Conclusion:*** Nobiletin stimulated transepithelial Cl^- secretion across human bronchial epithelia. The mechanisms involved activation of adenylate cyclase- and cAMP/PKA-dependent pathways, leading to activation of apical CFTR Cl^- channels.

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Introduction

Nobiletin is a citrus fruit-derived flavonoid isolated from the sweet orange peel of tangerines and from bitter orange peel [1]. Flavonoids have been widely known to have protective roles in body health because they possessed multiple biological functions such

Wing-hung Ko, PhD, Associate Professor

Room 607A, Lo Kwee-Seong Integrated Biomedical Sciences Building, The Chinese University of Hong Kong, Shatin, N.T., (Hong Kong)
Tel. 852-39436781, Fax 852-26035123, E-Mail whko@cuhk.edu.hk

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as anti-atherogenic, anti-tumor, and anti-inflammatory activities [2, 3]. For example, nobiletin reduced the risk of coronary heart disease and atherosclerosis at vascular walls by inhibiting macrophage foam cell formation [4]. Flavonoids also possessed anticancer effects [5] that resulted from their antioxidant activities. The flavonoids inhibited reactive oxygen species-induced damage and suppressed free radical generation [2]. In addition, the anti-inflammatory effects of flavonoids have been well studied. For example, nobiletin suppressed the activities and formation of inflammatory metabolites such as cyclooxygenase-2 (COX-2) inducible nitric oxide (NO) synthase and prostaglandin E_2 present during skin inflammation [6]. These effects prevented carcinogenesis and abnormal cell proliferation. Therefore, it has been suggested that nobiletin has novel functions, including a role as a possible chemopreventive agent in inflammation-associated tumorigenesis [6].

Several naturally occurring flavonoids, including quercetin, tangeretin, and baicalein, have been shown to stimulate Cl⁻ secretion in different epithelia, such as nasal and intestinal epithelia [7-10]. Despite the relatively large number of studies characterizing the effects of flavonoids on epithelial ion transport, there has been only one report showing that nobiletin can stimulate Cl⁻ secretion in human colonic T84 cells, possibly via a cAMP-dependent pathway involving cystic fibrosis transmembrane conductance regulator (CFTR) [11]. Furthermore, there has currently been no report describing a prosecretory effect of nobiletin on airway epithelial electrolyte transport function, although nobiletin was reported to activate CFTR in Fischer rat thyroid (FRT) epithelial cells stably transfected with human CFTR [12]. Notably, not all flavonoids possessed prosecretory effects on epithelia. Schuier et al. have also reported that cocoa-related flavonoids inhibited CFTR-mediated Cl⁻ transport in T84 colonic epithelia [13].

In traditional Chinese medicine, dry citrus fruit peels are widely used as remedies to alleviate coughs and reduce mucus secretion in the respiratory tract. It is believed that the major bioactive compounds are flavonoids (e.g., nobiletin, tangeretin) present in the peel of these fruits [1]. Recently, nobiletin has been reported to have antagonistic effects on eosinophilic airway inflammation of asthmatic rats [14]. Nobiletin also inhibited the IL-1β-induced expression of the proinflammatory protein, COX-2, in A549 human lung cells [15]. Thus, nobiletin has been predicted to comprise a new class of anti-asthmatic airway inflammatory drugs.

Epithelial damage and abnormal ion transport play pivotal roles during the inflammatory process. Stimulation of Cl⁻ secretion in airway epithelia helped maintain the thickness and composition of the airway surface liquid (ASL), which then affected airway mucus clearance [16, 17]. Stimulation of ion transport and the resultant osmotic efflux of water to the apical surface are parts of a natural defense system that functions to effectively remove noxious stimuli during inflammation or cellular damage, so it is important to investigate the effect of nobiletin on electrolyte transport across airway epithelia. The aim of the present study was therefore to examine the effects of nobiletin on basal short-circuit currents in the 16HBE14o- human bronchial epithelial cell line, and to characterize the signal transduction pathways that allowed nobiletin to regulate electrolyte transport.

Materials and Methods

Materials

Membrane permeant acetoxymethyl ester (AM) forms of Fura-2, Pluronic F127, and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). Forskolin, nobiletin, 4,4'-diisothiocyano-stilbene-2,2'-disulphonic acid (DIDS), clotrimazole, and 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34) were obtained from Sigma-Aldrich (St. Louis, MO, USA). H89 dihydrochloride, MDL-12330A, and CFTR$_{inh}$-172 were obtained from Calbiochem (La Jolla, CA, USA). Trans-6-cyano-4-(*N*-ethylsulphonyl-*N*-methylamino)-3-hydroxy-2,2-dimethyl-chromane (Chromanol 293B) was obtained from Tocris (Bristol, UK). All other general laboratory reagents were obtained from Sigma-Aldrich, and all cell culture reagents were obtained from Invitrogen.

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Cell culture

All experiments were performed using an immortalized cell line, 16HBE14o-, derived from bronchial surface epithelial cells [18], and a cystic fibrosis (CF) human airway epithelial cell line, CFBE41o- [19]. Cells were grown in media using standard culture techniques, as described previously [20, 21]. In brief, cells were maintained in Minimum Essential Medium with Earle's salts supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured on plastic flasks coated with fibronectin and collagen (BD Biosciences, San Jose, CA, USA) and were incubated in humidified 95% air-5% CO_2 at 37°C.

Measurement of short-circuit current (I_{SC})

Confluent 16HBE14o- cells were used to measure I_{SC} as described previously [9]. The monolayers were cultured on Transwell®-COL membranes (Costar, Cambridge, MA, USA) with a 0.4 µm pore diameter (culture area, 0.2 cm^2), mounted in an Ussing chamber, and bathed in normal bicarbonate-buffered Krebs-Henseleit (KH) solution with the following components (in mM): NaCl, 117; $NaHCO_3$, 25; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; and D-glucose, 11; the pH was 7.4 when bubbled with 5% CO_2/95% O_2. To generate a favorable gradient for Cl- passage, a basolateral-to-apical Cl- gradient was applied across the monolayers by changing the apical KH solution to a reduced Cl- concentration [18]. The low Cl- concentration solution (10 mM) was prepared by isosmotically replacing NaCl, KCl, $CaCl_2$, and $MgCl_2$ with Na-gluconate, K-gluconate, Ca-gluconate, and $MgSO_4$, respectively. The potential difference was clamped to 0 mV, and I_{SC} was simultaneously measured using a voltage clamp amplifier (VCC MC6; Physiologic Instruments, San Diego, CA, USA). A transepithelial potential difference of 1 mV was applied periodically, and the resultant change in the current was used to calculate the transepithelial resistance using Ohm's law. The cells reached confluence after 10 days, with a resistance greater than 150 $\Omega \cdot cm^2$.

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

Calcium signals in cells grown on glass coverslips were measured as previously described [21, 22]. Briefly, cells were loaded with Fura-2 by incubation (45 minutes, 37°C) in KH solution containing 3 µM Fura-2-AM and 1.6 µM Pluronic F127. The Fura-2-loaded cells were washed with KH solution, and the entire coverslip was then transferred to a closed perfusion chamber mounted on the stage of an inverted microscope (Nikon TE300; Nikon, Tokyo, Japan). The cells were viewed with a 40× extra-long working distance objective (Nikon CFI Plan Fluor ELWD, 0.6 numerical aperture) and the Fura-2 fluorescence ratios recorded (PTI Ratio-Master fluorescence system; Photon Technology International, Edison, NJ, USA) from an optical field containing 30 – 40 cells in the center of the epithelium. Fura-2 ratios were used to represent changes in $[Ca^{2+}]_i$ using *Felix* software (Photon Technology International).

Real-time monitoring of cAMP by Fluorescence Resonance Energy Transfer (FRET)

Real-time cAMP changes in 16HBE14o- cells were monitored using CFP-Epac-YPF, an Epac (Exchange Protein directly activated by cAMP)-based polypeptide FRET reporter [23]. FRET imaging experiments were performed using the MetaFluor Imaging System with FRET module (Molecular Devices, Downingtown, PA, USA) as described previously [24, 25]. In brief, 16HBE14o- cells were transfected with the Epac-based cAMP sensor with Lipofectamine 2000 according to the manufacturer's protocol. Cells on glass coverslips were placed on the inverted microscope (Olympus IX70; Olympus, Center Valley, PA, USA) equipped with a 40× water immersion objective (numerical aperture 0.6) and excited at 436 nm. CFP and YFP images were simultaneously recorded by the imaging setup equipped with the Photometrics DV^2 emission splitting system (Photometrics, Tucson, AZ, USA) including two emission filters (470/30 nm for CFP and 535/30 nm for FRET), and a scientific CMOS camera (pco.edge 5.5; PCO AG, Kelheim, Germany). Acquired fluorescence images were background subtracted, and real-time cAMP changes were represented by a normalized CFP/FRET emission ratio similar to that described by Li et al. [26]. Images were digitized and analyzed using MetaFluor Imaging Software (v.7.5).

Simultaneous measurement of cAMP and I_{SC}

Nobiletin-induced FRET signals and I_{SC} changes were measured simultaneously in polarized epithelia, similar to that described for simultaneous measurements of $[Ca^{2+}]_i$ and I_{SC} [21]. Briefly, cells were transfected with FRET reporter using the NEON® transfection system and transfection kit (Invitrogen) before they were

grown on a Transwell®-COL membrane. Membranes bearing transfected epithelia were then mounted in a miniature Ussing chamber. The cells were initially perfused bilaterally with normal KH solution, then the apical KH solution was changed to a solution containing low chloride ion concentration. Real-time changes in cAMP by FRET were monitored as described above. The I_{SC} was simultaneously measured using a voltage clamp amplifier (VCC 600; Physiologic Instruments). Both signals were digitized and recorded using the MetaFluor Imaging Software.

Data analyses

Pooled data were expressed as means ± standard errors (S.E.), and values of n referred to the number of experiments in each group. Experimentally induced changes in the Fura-2 fluorescence ratio, FRET ratio, and I_{SC} were quantified by measuring each parameter at the peak of a response and subtracting the equivalent values measured immediately prior to stimulation. Statistical comparisons between control and treated epithelia were performed using Student's t-test with P < 0.05 considered significant.

Results

Effect of apical vs. basolateral nobiletin on baseline I_{SC}

When clamped in the Ussing chamber, the 16HBE14o- epithelia exhibited a basal I_{SC} of 28.8 ± l.6 μA/cm² and a transepithelial resistance of 295.8 ± 25.2 Ω·cm² (n = 24). To examine the effect of nobiletin upon I_{SC} in the 16HBE14o- cell line, epithelial cells were stimulated with apical or basolateral application of nobiletin (100 μM). As shown in Fig. 1A and C, both apical and basolateral applications resulted in an increase of I_{SC}. Apical application of nobiletin stimulated an I_{SC} response with a larger magnitude when compared to that of basolateral application. Responses to both apical and basolateral nobiletin were concentration-dependent as shown in Fig. 1B and D, with apparent EC$_{50}$ of 14.5 μM and 9.7 μM, respectively. In another series of experiments, the I_{SC} responses were quantified in control epithelia and in epithelia pretreated with apical or basolateral application of nobiletin in the contralateral membrane domain. Basolateral treatment with a maximal concentration of nobiletin (100

Fig. 1. Effect of nobiletin on I_{SC} response on the 16HBE14o- human bronchial epithelial cell line. The epithelia were stimulated with apical (ap) (A) or basolateral (bl) (C) application of nobiletin. Transient current pulses were the results of intermittently clamping the potential at 1 mV. The dashed lines represent zero I_{SC}. (B) and (D) show the concentration-dependent increases in I_{SC} stimulated by apical (B) or basolateral (D) application of nobiletin. Data represent the mean ± S.E.M. for 11 – 13 separate epithelia.
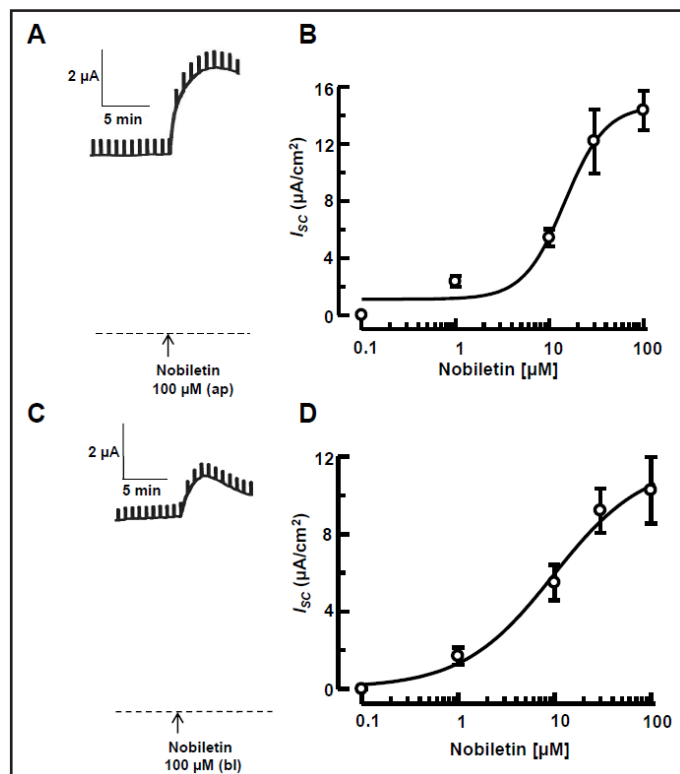
Fig. 2. Cross-desensitization between apical and/or basolateral application of nobiletin for I_{SC} of the 16HBE14o- human bronchial epithelial cell line. (A) Nobiletin was applied sequentially to the basolateral and then apical sides of the epithelia as indicated. (B) Changes in I_{SC} stimulated by apical application of nobiletin were quantified in control epithelia and in epithelia pretreated with contralateral application of nobiletin. Using an identical protocol, nobiletin was applied sequentially to the apical and then basolateral sides of the epithelia as indicated (C) and the summarized data are shown in (D). In (A) and (C), transient current pulses were the results of intermittently clamping the potential at 1 mV. The dashed lines represent zero I_{SC}. Each column represents the mean ± S.E.M. for 4 – 9 separate epithelia. *P < 0.05 compared with the control.
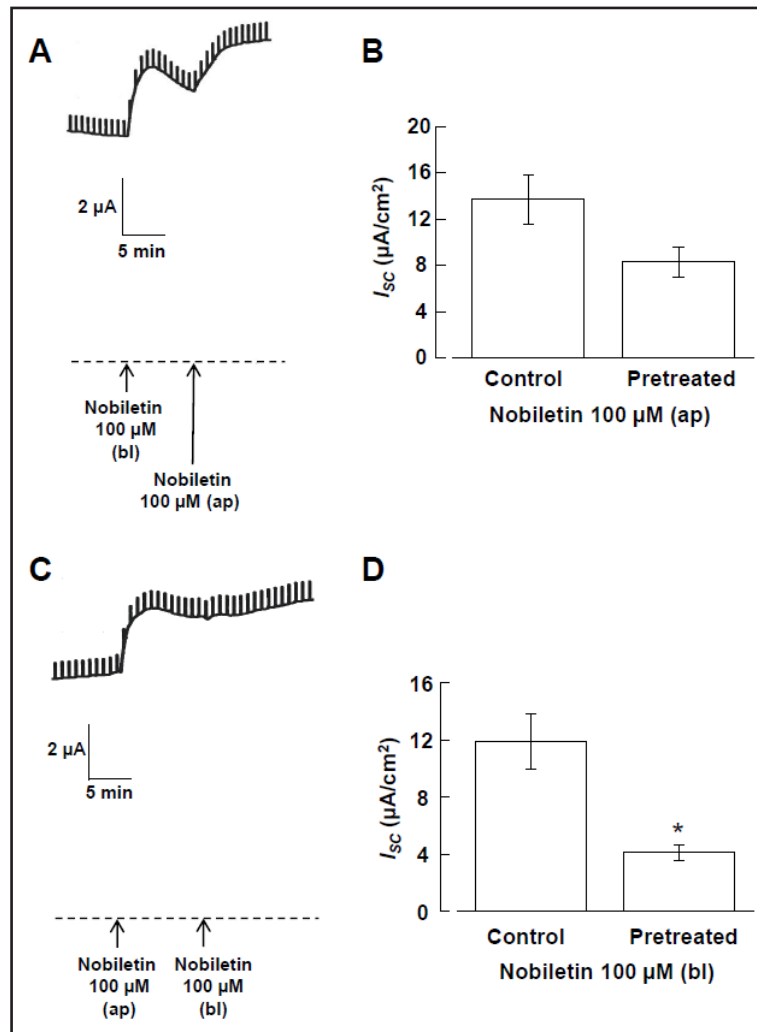

Fig. 3. Effect of ion substitution on nobiletin-stimulated I_{SC} of the 16HBE14o- human bronchial epithelial cell line. Summarized data show the effect of apical or basolateral application of nobiletin on epithelia bathed in normal Krebs-Henseleit (KH) solution and Cl⁻-free solution. Each column represents the mean ± S.E.M. for 4 – 5 separate epithelia. *P < 0.05 compared with the control.
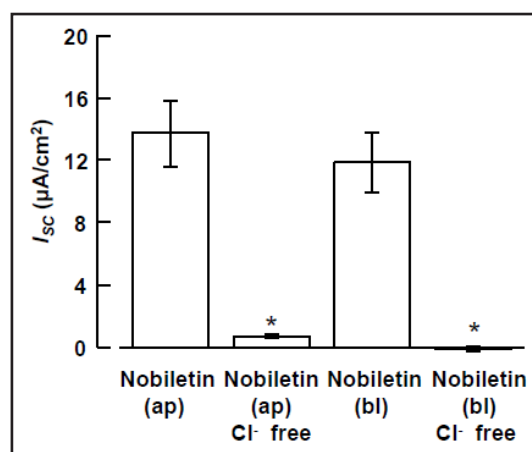
μM) did not alter the subsequent I_{SC} responses to apical addition of nobiletin (100 μM; P = 0.108) (Fig. 2A and B). In contrast, pretreating the epithelia with apical nobiletin reduced the I_{SC} responses to basolateral addition of nobiletin (P = 0.009) (Fig. 2C and D). The replacement of apical normal KH solution by a low Cl⁻ concentration solution generated a favorable serosal to the mucosal Cl⁻ gradient, and the increase in I_{SC} response could be due to Cl⁻ secretion as shown in our previous studies [21, 25, 27]. To confirm that nobiletin-stimulated I_{SC} responses were due to Cl⁻ secretion, ion substitution experiments were performed. Both apical and

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Fig. 4. Effect of Cl⁻ channel blockers on nobiletin-stimulated I_{SC} of the 16HBE14o- human bronchial epithelial cell line. The epithelia were treated apically with Cl⁻ channel blockers 4,4'-diisothiocyano-stilbene-2,2'-disulphonic acid [DIDS; 300 μM, (A) and (B)] or CF-TR$_{inh}$-172 [(10 μM, (C) and (D)]. After the basal I_{SC} had stabilized, the epithelia were stimulated with apical [(A) and (C)] or basolateral [(B) and (D)] application of nobiletin. Transient current pulses were the results of intermittently clamping the potential at 1 mV. The dashed lines represent zero I_{SC}. (E) and (F) Summarized data showing the effect of Cl⁻ channel blockers on apical or basolateral I_{SC} evoked by nobiletin. Each column represents the mean ± S.E.M. for 4 – 7 separate epithelia. *P < 0.05 compared with the control (nobiletin alone).
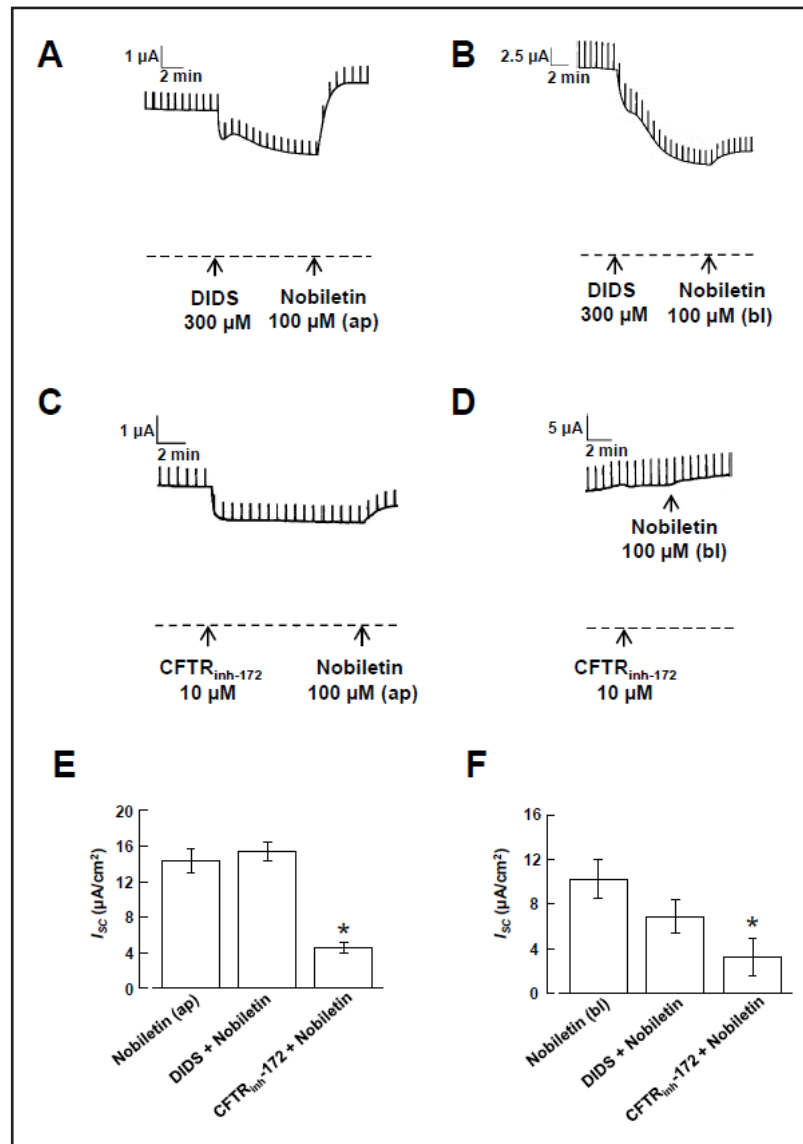

basolateral I_{SC} responses to nobiletin were abolished when Cl⁻ ions were substituted by gluconate (Fig. 3). In the presence of 10 μM forskolin, which maximally activated the cAMP-dependent CFTR Cl⁻ channels, the addition of nobiletin (100 μM) to the apical or basolateral membrane could still increase I_{SC} by 12.03 ± 1.18 μA/cm² and 9.69 ± 1.38 μA/cm² (n = 4), respectively. Taken together, the results showed that both apical and basolateral nobiletin was able to stimulate Cl⁻ secretion in human bronchial epithelial cells.

Effect of apical Cl⁻ channel blockers on nobiletin-stimulated I_{SC}

To investigate which apical Cl⁻ channel was involved in mediating the nobiletin-stimulated I_{SC} responses, different Cl⁻ channel inhibitors were used, including DIDS (300 μM) and CFTR$_{inh}$-172 (10 μM), which blocked calcium-activated chloride channels (CaCC) and CFTR, respectively [28, 29]. The epithelia were stimulated with apical or basolateral nobiletin in the absence (tracing not shown) or presence of DIDS (Fig. 4A and B) or CFTR$_{inh}$-172 (Fig. 4C and D). Summarized data are shown in Fig. 4E and F. Pretreating the epithelia with DIDS had no effect on the responses caused by apical (P = 0.493) or basolateral (P = 0.316) nobiletin. However, in the presence of CFTR$_{inh}$-172, the I_{SC} responses to apical and basolateral nobiletin were reduced by 67.2 ± 4.5% (P = 0.002) and 68.7 ± 16.3% (P = 0.016), respectively. These

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Fig. 5. Effect of K$^+$ channel blockers on nobiletin-stimulated I_{SC} in the 16HBE14o- human bronchial epithelial cell line. The epithelia were treated basolaterally with K$^+$ channel blockers Chromanol 293B [10 μM, (A) and (D)], TRAM-34 [10 μM, (B) and (E)], or clotrimazole [100 μM, (C) and (F)]. After the basal I_{SC} had stabilized, the epithelia were stimulated with apical [(A) – (C)] or basolateral [(D) – (F)] application of nobiletin. Transient current pulses were the result of intermittently clamping the
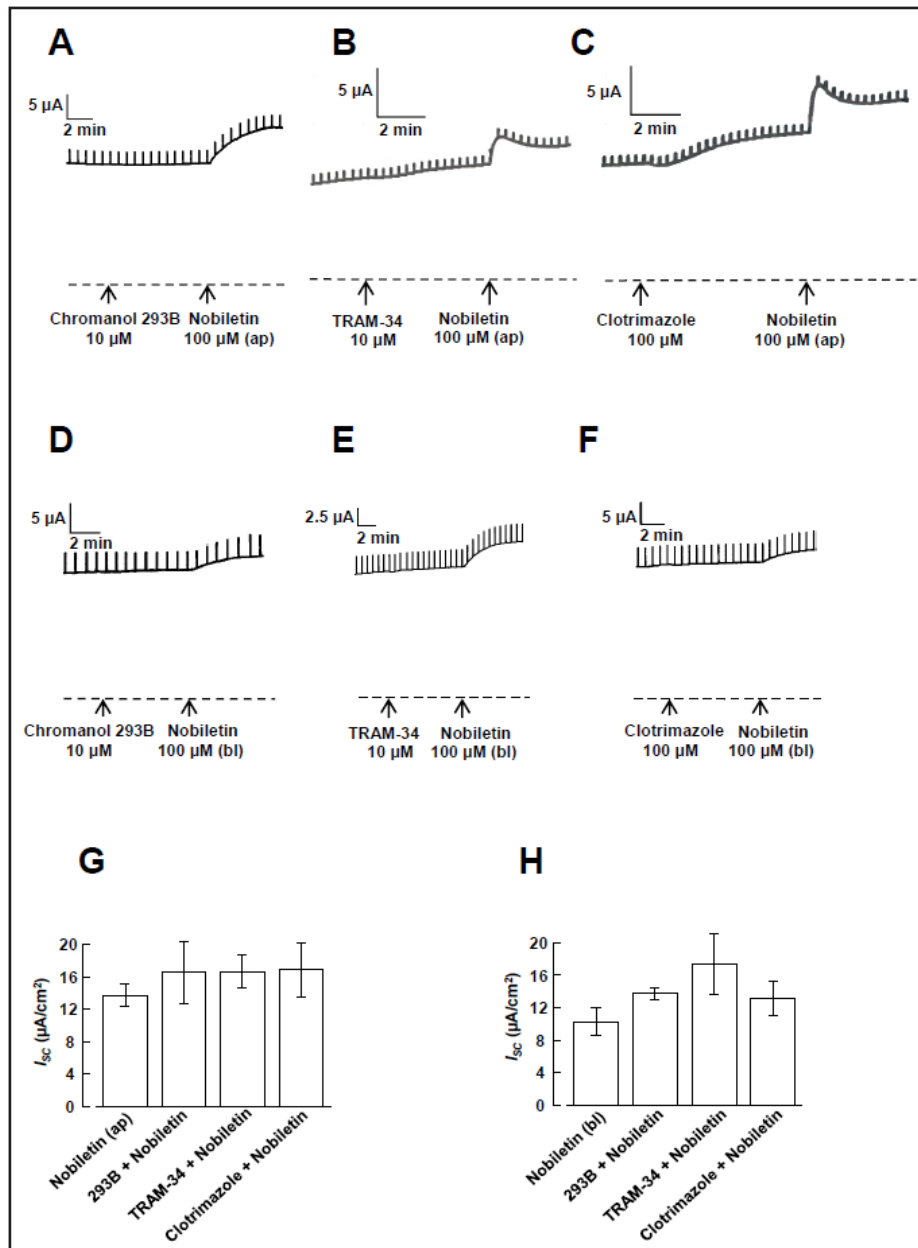
potential at 1 mV. The dashed lines represent zero I_{SC}. Summarized data showing the changes in I_{SC} induced by apical (G) or basolateral (H) application of nobiletin alone and in the presence of K$^+$ channel blockers. Each column represents the mean ± S.E.M. for 4 – 6 separate epithelia.

results suggested that nobiletin-stimulated Cl$^-$ secretion could be mediated through CFTR, but not CaCC. The contributions of CaCC and CFTR to the basal I_{SC} were 39.8 ± 5.2% and 21.2 ± 4.7%, respectively.

Effect of basolateral K$^+$ channel blockers on nobiletin-stimulated I_{SC}
Basolateral K$^+$ channels were important in providing the driving force for Cl$^-$ secretion via various apical Cl$^-$ channels [30]. Besides examining the involvement of apical Cl$^-$ channels, the sensitivity of nobiletin-stimulated Cl$^-$ secretion to basolateral K$^+$ channel inhibitors was also tested. The epithelia were pretreated with different basolateral K$^+$ channel inhibitors which included Chromanol 293B (Fig. 5A and D), TRAM-34 (Fig. 5B and E), and clotrimazole (Fig. 5C and F). Chromanol 293B was a cAMP-dependent K$^+$ channel (KCNQl, KvLQTl)
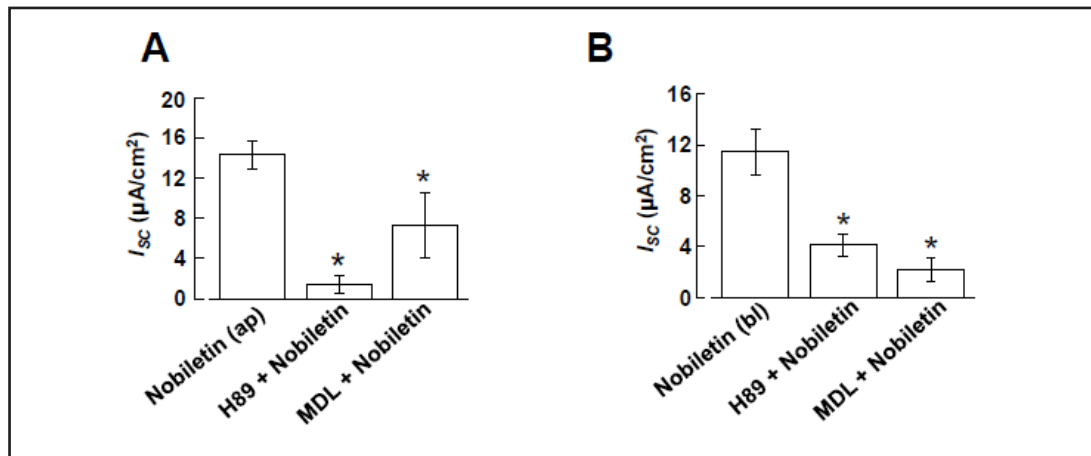
Fig. 6. Effect of protein kinase A (PKA) and adenylate cyclase (AC) inhibitors on nobiletin-stimulated I_{SC} in the 16HBE14o- human bronchial epithelial cell line. The epithelia were treated apically with PKA inhibitor H89 (1 μM) or AC inhibitor MDL-12330A (1 μM). After the basal I_{SC} had stabilized, the epithelia were stimulated with (A) apical or (B) basolateral application of nobiletin. Summarized data showing the effect of H89 or MDL-12330A on apical or basolateral I_{SC} evoked by nobiletin. Each column represents the mean ± S.E.M. for 4 – 5 separate epithelia. *$P < 0.05$ compared with the control (nobiletin alone).
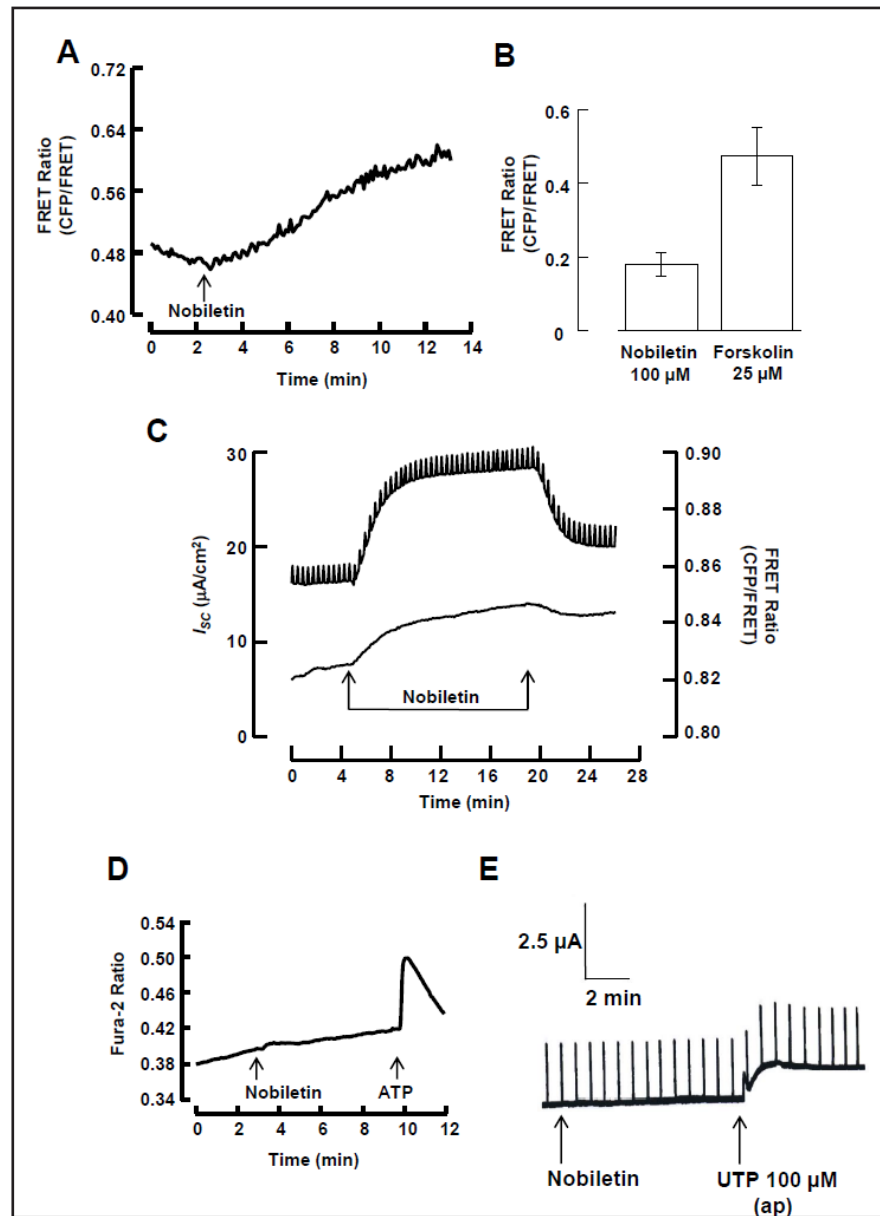
inhibitor [31]. TRAM-34 was a highly selective intermediate-conductance Ca^{2+}-dependent K^+ channel inhibitor, and clotrimazole was also a Ca^{2+}-dependent K^+ channel inhibitor [32]. The summarized data show that none of the K^+ channel inhibitors had an effect on apical (293B + nobiletin *vs* nobiletin, $P = 0.376$; TRAM-34 + nobiletin *vs* nobiletin, $P = 0.109$; clotrimazole + nobiletin *vs* nobiletin, $P = 0.311$) or basolateral (293B + nobiletin *vs* nobiletin, $P = 0.294$; TRAM-34 + nobiletin *vs* nobiletin, $P = 0.016$; clotrimazole + nobiletin *vs* nobiletin, $P = 0.409$) I_{SC} responses to nobiletin, suggesting that K^+ channels were not involved in mediating the nobiletin-stimulated Cl^- secretion.

Involvement of cAMP/protein kinase A (PKA)-, but not Ca^{2+}-dependent pathways, in nobiletin-stimulated I_{SC}

The above data suggested that CFTR was involved in the secretory pathway of nobiletin-stimulated Cl^- secretion. To further examine the signal transduction mechanism in activating apical CFTR, the involvement of PKA and adenylate cyclase (AC) were examined. The 16HBE14o- epithelia were first incubated with a PKA inhibitor, H89 (1 μM), at the apical side for 1 hour before the cells were stimulated with apical or basolateral nobiletin. The basal I_{SC} remained stable in the presence of H89. Figure 6 shows that the apical and basolateral nobiletin-stimulated I_{SC} responses were reduced by 90.2 ± 6.5% and 53.6 ± 12.3%, respectively. When the epithelia were incubated with an AC inhibitor, MDL-12330A, for 15 minutes on the apical side, the apical and basolateral nobiletin-evoked I_{SC} responses were reduced by 48.6 ± 6.5% and 80.9 ± 8.2%, respectively. These results indicated that a cAMP/PKA-dependent pathway was involved in the CFTR-mediated Cl^- secretion in 16HBE14o- cells.

Nobiletin-stimulated I_{SC} was sensitive to both PKA and AC inhibitors, suggesting that nobiletin stimulated cAMP formation. In the present study, imaging of real-time increases in cellular cAMP levels in 16HBE14o- cells was accomplished by a FRET-based approach using an Epac sensor. Figure 7A shows the tracing of the nobiletin-stimulated increase in the FRET ratio, representing the real-time changes in cAMP levels. Stimulation of cells with nobiletin increased the FRET ratio to 0.179 ± 0.032 (Fig. 7B). Addition of forskolin (an AC activator), which served as a positive control, robustly increased the FRET ratio to 0.473 ± 0.079. The mean increase of the nobiletin-stimulated FRET ratio relative to forskolin was approximately 38%. Together, the results further confirmed that stimulation of 16HBE14o- cells with nobiletin involved cAMP signaling.

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Fig. 7. Nobiletin stimulated a real-time increase in intracellular cAMP but not Ca^{2+} in the 16HBE14o- human bronchial epithelial cell line. (A) Real-time cAMP changes (FRET ratio) recorded in cells stimulated with 100 μM nobiletin. The summarized data are shown in (B). Forskolin (25 μM) was used as a positive control to stimulate AC. Each column represents the mean ± S.E.M. (n = 4). (C) Simultaneous measurements of real-time cAMP and I_{SC} induced by apical application of nobiletin. Epithelia were perfused with normal KH solution (basolateral) or low Cl^- concentration solution (apical) while changes in I_{SC} (upper trace) and FRET ratio (lower trace) were recorded. Nobiletin (100 μM) was added to the apical side as indicated. Transient current pulses were the result of intermittently clamping the potential at 1 mV. (D) Effect of nobiletin on $[Ca^{2+}]_i$ of 16HBE14o- cells. Representative recording showing ATP (100 μM), but not nobiletin (100 μM), stimulating an increase in the Fura-2 ratio (n = 4). (E) Representative recording showing UTP (100 μM), but not nobiletin (100 μM), stimulating an increase in I_{SC} of CFBE41o- cells (n = 3).

To further support the concept that the increase in I_{SC} was due to a nobiletin-stimulated cAMP signaling pathway, a simultaneous measurement of the I_{SC} and FRET ratio was performed in polarized 16HBE14o- epithelia. Application of apical nobiletin (100 μM) to 16HBE14o- epithelial cells grown on a Transwell®-col membrane stimulated an increase in I_{SC} of 16.0 ± 2.4 μA/cm² (Fig. 7C, upper trace). This was followed by a concomitant increase in the FRET ratio (Fig. 7C, lower trace). The increase in I_{SC} and FRET ratios were abolished when the epithelia were not perfused with nobiletin-containing solution, further supporting the earlier conclusion that nobiletin-stimulated Cl^- secretory responses were cAMP-dependent. Application of nobiletin did not stimulate any increase in the Fura-2 ratio, while the calcium-mobilizing agonist ATP stimulated an increase in the Fura-2 ratio of 0.075 ± 0.006 (Fig. 7D).

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To further confirm that the nobiletin-stimulated Cl- secretion was due to the activation of CFTR, the CF cell line (CFBE41o-), which lacked functional CFTR, was used. In Fig. 7E, no increase in I_{SC} could be observed after the application of apical nobiletin (100 μM). For CFBE41o- cells grown on glass coverslips, addition of 100 μM nobiletin also increased intracellular cAMP levels (FRET ratio = 0.021 ± 0.003, n = 5), similar to that observed in normal 16HBE14o- cells. However, the application of a calcium-mobilizing agent, UTP [21], stimulated an increase in I_{SC}, which was due to the activation of CaCC [33]. These results were consistent with our previous findings showing that nobiletin stimulated Cl- secretion through activation of CFTR.

Discussion

Citrus fruit-derived flavonoids have recently become widely studied because of their beneficial biological functions, including their anti-atherogenic, anti-atherosclerotic, anti-tumor, anticancer, and anti-inflammatory activities [2, 3]. Nobiletin is a citrus fruit-derived flavonoid isolated from tangerines [1], which may become a new class of anti-asthmatic and anti-inflammatory drugs for treatment of airways [14]. However, little is known about its effect on ion transport regulation in airway epithelial cells.

In the present study, nobiletin induced a concentration-dependent Cl- secretory response in 16HBE14o- cells at both apical and basolateral membranes. Because transepithelial Cl- secretion required the stimulation of apical Cl- channels and/or basolateral K+ channels, the involvement of different ion channels in nobiletin-stimulated Cl- secretion was examined with various selective ion channel inhibitors. First, DIDS, a CaCC inhibitor [34] and CFTR$_{inh}$-172, a potent selective inhibitor of CFTR [35], were used to study the involvement of the two major types of Cl- channels expressed in airway epithelia [36]. The apical and basolateral nobiletin-stimulated I_{SC} was sensitive to CFTR$_{inh}$-172, but insensitive to DIDS. These results indicated that nobiletin might stimulate the Cl- secretion by activating CFTR instead of CaCC. However, it should be noted that DIDS also inhibits volume-sensitive Cl- channels, but has no effect on CFTR [37]. Therefore, we could not exclude the possibility that nobiletin might stimulate volume-sensitive Cl- channels. In addition, CFTR$_{inh}$-172 might also exert a non-specific effect on reactive oxygen species production, independently of CFTR inhibition [38]. In contrast, the apical and basolateral nobiletin-stimulated I_{SC} was completely insensitive to three different types of K+ channel inhibitors added to the basolateral side of the epithelia, showing that cAMP-dependent and Ca^{2+}-dependent basolateral K+ channels were not involved in driving nobiletin-stimulated Cl- secretion. Addition of these three different types of K+ channel inhibitors also did not produce any significant inhibition on apical or basolateral nobiletin-induced I_{SC} (n = 3 – 6; data not shown). This is in contrast with the P2Y receptor-mediated Cl- secretion in 16HBE14o- cells, in which the basolateral Ca^{2+}-dependent K+ channels were important in driving the apical Cl- exit, because the nucleotide-stimulated I_{SC} was inhibited by both TRAM-34 and clotrimazole [21]. Similar to our previous findings, cAMP-dependent K+ channels were also not involved in mediating the I_{SC} increase, despite the observation that both UDP [21] and nobiletin could raise intracellular cAMP levels. It appeared that although cAMP-dependent K+ channels were expressed in 16HBE14o- cells [39], they were not accessible to cAMP-dependent signaling molecules. It has been shown that in Calu-3 cells, the cAMP effects were limited to short distances from the receptor that were coupled to the generation of cAMP. This may be due to the compartmentalization of individual phosphodiesterase isozymes that formed a diffusion barrier to confine the cAMP signaling to localized functional domains, including CFTR Cl- channels within the cells [40].

In the present study, nobiletin stimulated a rise in cAMP levels, which was capable of activating PKA and CFTR Cl- channels, leading to Cl- secretion. CFBE41o- cells that lacked functional CFTR were used for further evaluation of the involvement of CFTR in the stimulatory effect of nobiletin in airway epithelial cells. The apical addition of nobiletin in CF cells did not stimulate any I_{SC} response. However UTP, which increased [Ca^{2+}]$_i$ in 16HBE14o- cells [21],

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stimulated Cl⁻ exit via apical CaCC. These results further confirmed that CFTR was required for nobiletin-stimulated Cl⁻ secretion. In FRT cells stably transfected with CFTR, nobiletin stimulated the channel activity in a dose- and time-dependent manner [12]. A similar stimulatory effect on CFTR channel activity was also observed by another citrus-derived flavonoid, tangeretin [7]. Because of its therapeutic potential in treating CF [41], the best-known and studied flavonoid, which can stimulate CFTR-dependent Cl⁻ secretion, is genistein [42]. Genistein is a potent activator of CFTR and ΔF508 CFTR in human airway epithelial cell lines [43]. However, its molecular mechanism is complex. Some studies reported that cAMP-dependent phosphorylation of CFTR is required for the stimulatory effect of genistein [44, 45]. Low micromolar concentrations of genistein potentiate CFTR channel gating, whereas higher concentrations inhibit CFTR gating and weakly inhibit Cl⁻ flow through the CFTR pore [46, 47]. In the present study, pre-activation of the cAMP/PKA signaling pathway was not required. Although there was significant basal I_{SC} before nobiletin stimulation, H89 did not have an inhibitory effect on basal I_{SC}, suggesting that the cAMP/PKA signaling pathway was not activated. In the presence of 10 μM forskolin, which maximally activated the cAMP-dependent CFTR Cl⁻ channels, addition of nobiletin further increased I_{SC}. Like genistein, these results suggested that nobiletin may potentiate CFTR activity elicited with forskolin. However, the detailed molecular mechanism by which nobiletin increases CFTR activity is still unclear, and awaits further investigation using the patch-clamp technique.

It was well known that the activation of CFTR resulted from a cAMP-mediated signaling pathway [48]. Our results showed that nobiletin-stimulated Cl⁻ secretion in 16HBE14o- cells required the activation of CFTR. Therefore, the involvement of AC and the downstream signaling molecule of cAMP, PKA, were examined. By pretreating the epithelia with either H89 or MDL-12330A, the apical and basolateral I_{SC} responses of nobiletin were significantly reduced, indicating that PKA and AC were necessary for the nobiletin-stimulated Cl⁻ secretory response. Taken together, nobiletin activated AC which increased intracellular cAMP levels, followed by PKA activation, leading to stimulation of CFTR activity and Cl⁻ secretion. Notably, the nobiletin-stimulated increase in cAMP level was able to activate the apical CFTR, but not the basolateral cAMP-dependent K⁺ channels. This may be due to the localization and restricted properties of cAMP, notably its inability to diffuse freely throughout the cell [49, 50]. Because both AC and PKA are in the vicinity of apical CFTR, low levels of cAMP in the sub-plasma membrane are therefore capable of stimulating PKA, and then CFTR activity. This may explain why H89 could completely inhibit the apical effect of nobiletin when compared to that of MDL-12330A. For basolateral nobiletin, the inhibitory effects of H89 and MDL-12330A were more comparable. However, the detailed molecular mechanism underlying the differential inhibitory effects of H89 and MDL-12330A on apical and basolateral nobiletin effects remains obscure and needs further investigation. In the present study, apical application of nobiletin stimulated a larger increase in I_{SC} response than that of basolateral application of nobiletin, which may be due to receptors and/or signaling molecules for nobiletin stimulation located mainly on the apical portion of the epithelial cells. Thus, the apical application of nobiletin stimulated a larger response in epithelial cells. Another possibility was that the lipophilic citrus flavonoid penetrated the apical membrane of 16HBE14o- cells more efficiently than the basolateral membrane. Moreover, pretreating the epithelia with basolateral nobiletin did not affect the apical nobiletin-stimulated I_{SC} response, but the reverse process did not result in the same outcome. Whether apical and basolateral nobiletin activated different pools of cAMP that were regulated by different phosphodiesterases, as seen in Madin-Darby canine kidney epithelial cells, will require further investigation [51].

In T84 cells, nobiletin stimulated an increase in I_{SC} via a cAMP-dependent pathway. However, by using a radioimmunoassay, which was an endpoint measurement approach, the flavonoid did not stimulate cAMP production to the extent seen with a vasoactive intestinal peptide [52]. In the present study, using FRET-based simultaneous measurements, we confirmed that the cellular mechanism for the prosecretory effect of nobiletin was coupled to a real-time increase in intracellular cAMP levels. The cellular mechanism did not involve modulation of Ca²⁺ signaling pathways, because nobiletin did not evoke any increase in

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$[Ca^{2+}]_i$, and the Cl^- movement across the apical membrane did not occur via CaCC. Nobiletin did not stimulate Cl^- secretion in CFBE41o- cells, but this was not due to its inability to raise cAMP levels. To the best of our knowledge, this is the first report to demonstrate that it was feasible to simultaneously measure real-time changes in cAMP and I_{SC} in polarized epithelia. Using this technique, changes in cAMP levels and the time course for I_{SC} responses in epithelia could be better correlated. Furthermore, in neurons, it has been shown that nobiletin was capable of activating a cAMP/PKA-dependent signaling pathway [53].

Hydration of the normal airway surface was dependent on active ion transport processes of airway epithelia, which are highly water-permeable [54]. The coordinated regulation and balance of Cl^- secretion and Na^+ reabsorption of airway surfaces were important in maintaining the thickness and composition of the airway surface liquid (ASL), which then affected ciliary beating frequency and airway mucus clearance [16, 17]. It has been shown that nobiletin and tangeretin stimulated fluid secretion from submucosal glands isolated from mouse trachea [3, 7]. Fluid secretion not only affected the volume of ASL, but Veit et al. recently reported that the inducible expression of either CFTR or the Ca^{2+}-activated Cl^- channel, TMEM16A, attenuated the expression of proinflammatory cytokines in human CF bronchial epithelia [55]. As a consequence, the ion transport activity of airway epithelia was important for suppressing the secretion of proinflammatory cytokines. In CF, the disruption of this mechanism may contribute to excessive lung inflammation. Notably, the dried peels which contain nobiletin are widely used in traditional Chinese medicine to treat cough and asthma, because it is believed that it helps remove phlegm in the lung [32]. Therefore, it is possible that part of the anti-inflammatory effect of nobiletin in an asthmatic mouse model [14] was due to the prosecretory effect of nobiletin.

In conclusion, the present study demonstrated that nobiletin stimulated Cl^- secretion in 16HBE14o- human bronchial epithelial cells. The mechanism involved the activation of CFTR, which was mediated by a AC/cAMP/PKA-dependent signaling pathway. Based upon the results of our study, the use of citrus fruit-derived flavonoid may therefore become a potential therapeutic strategy for treating electrolyte transport disorders in airway epithelia.

Acknowledgments

Disclosure Statement

None declared.

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