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Full paper

Cellular mechanisms underlying the inhibitory effect of flufenamic acid on chloride secretion in human intestinal epithelial cells

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A B S T R A C T

Intestinal Cl^- secretion is involved in the pathogenesis of secretory diarrheas including cholera. We recently demonstrated that flufenamic acid (FFA) suppressed *Vibrio cholerae* El Tor variant-induced intestinal fluid secretion via mechanisms involving AMPK activation and NF-κB-suppression. The present study aimed to investigate the effect of FFA on transepithelial Cl^- secretion in human intestinal epithelial (T84) cells. FFA inhibited cAMP-dependent Cl^- secretion in T84 cell monolayers with IC_{50} of ~8 μM. Other fenamate drugs including tolfenamic acid, meclofenamic acid and mefenamic acid exhibited the same effect albeit with lower potency. FFA also inhibited activities of CFTR, a cAMP-activated apical Cl^- channel, and KCNQ1/KCNE3, a cAMP-activated basolateral K^+ channel. Mechanisms of CFTR inhibition by FFA did not involve activation of its negative regulators. Interestingly, FFA inhibited Ca^{2+}-dependent Cl^- secretion with IC_{50} of ~10 μM. FFA inhibited activities of Ca^{2+}-activated Cl^- channels and $K_{Ca}3.1$, a Ca^{2+}-activated basolateral K^+ channels, but had no effect on activities of Na^+–K^+–Cl^- cotransporters and Na^+–K^+ ATPases. These results indicate that FFA inhibits both cAMP and Ca^{2+}-dependent Cl^- secretion by suppressing activities of both apical Cl^- channels and basolateral K^+ channels. FFA and other fenamate drugs may be useful in the treatment of secretory diarrheas.

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1. Introduction

Secretory diarrheas including cholera result from over-stimulation of transepithelial Cl^- secretion, which can be classified into cAMP and Ca^{2+}-dependent processes.[1] Transepithelial Cl^- secretion requires functional cooperation between several types of transport proteins including Cl^- channels in the apical membrane and K^+ channels, Na^+–K^+ ATPases and Na^+–K^+–Cl^- cotransporters (NKCC) in the basolateral membrane.[1] The cAMP and Ca^{2+}-dependent Cl^- secretion involves different types of apical Cl^- channels and basolateral K^+ channels, namely, cystic fibrosis transmembrane conductance regulator (CFTR) and calcium-activated Cl^- channel (CaCC), and KCNQ1/KCNE3 and $K_{Ca}3.1$ K^+ channels, respectively.[2–5] Cl^- is taken up via NKCC and exited via apical Cl^- channels. Na^+–K^+ ATPases and basolateral K^+ channels function to maintain the sustained driving forces for Cl^- secretion. Our research group recently demonstrated that CFTR and CaCC-mediated Cl^- secretion similarly contributes to the intestinal fluid secretion in a mouse closed loop model of cholera induced by infection with *Vibrio cholerae* El Tor variant (EL), a causative agent of current cholera outbreaks in several regions.[6] In addition, intestinal barrier disruption was observed in the EL-infected intestinal loops. Inhibition of nuclear factor kappa B (NF-κB) and cyclooxygenase-2 (COX-2) markedly abrogated both intestinal fluid secretion and barrier disruption induced by EL.[6]

Flufenamic acid (FFA), a non-steroidal anti-inflammatory drug (NSAID), is used in the treatment of inflammatory conditions

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including pain and pyroxia. Accumulated lines of evidence reveal an additional role of FFA as a functional modulator of several types of ion channels, e.g. an inhibitor of transient receptor potential channels[7] and voltage-gated sodium channels[8] and an activator of Na^+-activated K^+ channel Slo2.1.[9] Interestingly, we recently found that FFA significantly prevented the EL-induced intestinal fluid secretion and barrier disruption in mice via mechanisms involving AMP-activated protein kinase (AMPK) activation and suppression of NF-κB-mediated inflammatory responses.[10] However, the effect of FFA on intestinal Cl^- secretion, which also participates in the pathogenesis of cholera, has never been investigated. Therefore, this study aimed to investigate the effect of FFA on both cAMP and Ca^{2+}-dependent Cl^- secretion in human intestinal epithelial cells and its underlying mechanisms using T84 cells, a crypt-like colorectal cancer cell line, as a model of human intestinal epithelium.

2. Materials and methods

2.1. Materials

Fenamates and their derivatives including flufenamic acid, tolfenamic acid, meclofenamic acid, mefenamic acid, diphenylamine, *N*-phenylanthranilic acid, and 3-(trifluoromethyl)aniline were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Fetal bovine serum (FBS), Trypsin, penicillin and streptomycin were purchased from HyClone (Logan, UT, USA). DMEM F-12 cultured medium was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Calbiochem (San Diego, California, USA). Snapwell inserts were purchased from Corning-Costar Corp (Corning, NY, USA).

2.2. Cell culture

T84 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). They were cultured in DMEM F-12 medium supplemented with 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator under an atmosphere of 95% O_2/5% CO_2 at 37 °C.

2.3. Electrophysiological analyses

Transepithelial ion transport across T84 cell monolayers grown on a Snapwell insert (transepithelial electrical resistance >1000 Ω cm^2) was determined by the Ussing chamber techniques. For short-circuit current (I_{sc}) analysis, both apical and basolateral hemichambers were filled with Kreb's solutions (pH 7.3) containing NaCl (120 mM), $NaHCO_3$ (25 mM), KH_2PO_4 (3.3 mM), K_2HPO_4 (0.8 mM), $MgCl_2$ (1.2 mM), $CaCl_2$ (1.2 mM) and glucose (10 mM). For apical Cl^- current (I_{Cl^-}) measurements, apical and basolateral hemichambers were filled with low Cl^- and high Cl^- buffers to create a basolateral-to-apical Cl^- gradient. The basolateral buffer (pH 7.3) contained NaCl (130 mM), KCl (2.7 mM), KH_2PO_4 (1.5 mM), $CaCl_2$ (1 mM), $MgCl_2$ (0.5 mM), Na-HEPES (10 mM) and glucose (10 mM). In apical buffer (pH 7.3), 65 mM of NaCl was substituted with 65 mM sodium gluconate, and the $CaCl_2$ concentration was 2 mM. In addition, amphotericin B (250 μg/mL) was added into the basolateral buffers to enable basolateral membrane permeabilization. To determine the cAMP- and Ca^{2+}-activated basolateral K^+ current (I_{K^+}), K^+ gradient buffers were used to create the apical-to-basolateral K^+ gradient. The apical high K^+ buffer (pH 7.3) contained K^+-gluconate (142.5 mM), $CaCl_2$ (1.25 mM), $MgSO_4$ (0.40 mM), KH_2HPO_4 (0.43 mM), Na_2HPO_4 (0.35 mM), Na-HEPES (10 mM) and glucose (5.6 mM). In the basolateral low K^+ buffer (pH 7.3), K^+-gluconate concentration was reduced to 5.4 mM and replaced with *N*-methylglucamine (136.9 mM). In addition, amphotericin B (250 μg/mL) was added into the apical solution for apical membrane permeabilization and ouabain (1 mM) was added into the basolateral solution to inhibit Na^+–K^+ ATPase activity. I_{sc}/I_{Cl^-}/I_{K^+} was measured using a VCC-600 voltage/current clamp (Physiologic Instruments, San Diego, CA, USA) with an Ag/AgCl electrode and a 3 M KCl agar bridge.

2.4. Measurement of Na^+–K^+ ATPase activity

To determine Na^+–K^+ ATPase activity in T84 cells, ouabain-sensitive I_{sc} was analyzed as previously described.[11] In brief, apical and basolateral chambers were filled symmetrically with Kreb's solutions. Thereafter, DMSO or FFA was added into the basolateral chamber followed by apical membrane permealization by amphotericin B. After the amphotericin B-elicited I_{sc} was stabilized, ouabain was added into the basolateral chamber. The ouabain-sensitive I_{sc} was used as an indicator of Na^+–K^+ ATPase activity.

2.5. Measurement of NKCC activity

The NKCC activity in T84 cells was determined using thallium (Tl^+) influx-based fluorescent assays (Invitrogen, Carlsbad, California, USA) as previously described.[12]

2.6. Data analysis and statistical methods

All data are represented as means ± S.E.M. Student's *t* test was used to determine the differences between control and treatment groups, with a *p*-value of <0.05 being considered statistically significant.

3. Results

3.1. Effect of FFA and other fenamates on cAMP-dependent Cl^- secretion

First, we determined the effect of FFA and other fenamates including tolfenamic acid (TFA), meclofenamic acid (MCFA) and mefenamic acid (MFA) (Fig. 1A) on cAMP-dependent Cl^- secretion, which was stimulated by forskolin (adenylate cyclase activator), in T84 cell monolayers using I_{sc} measurements. As shown in Fig. 1B and C, FFA dose-dependently inhibited the cAMP-dependent Cl^- secretion with IC_{50} of ~8 μM and complete inhibition at 50 μM. The cAMP-dependent Cl^- secretion was also inhibited by TFA, MCFA and MFA with IC_{50} of ~26 μM, 23 μM and 100 μM, respectively. Data of diclofenac was included for comparison (IC_{50} of ~20 μM). This result indicates that FFA exhibits the highest potency in inhibiting cAMP-dependent Cl^- secretion in T84 cells.

3.2. CFTR inhibition by FFA

To investigate the effect of FFA on CFTR channel activity, apical I_{Cl^-} analysis was performed in T84 cell monolayers. As shown in Fig. 2A and Fig. 2B, FFA dose-dependently inhibited the CFTR-mediated I_{Cl^-} stimulated by three different CFTR agonists including forskolin, CPT-cAMP (non-hydrolysable cell-permeable cAMP analog) and genistein (direct CFTR activator) with similar IC_{50} of ~8 μM.

CFTR channel activity is suppressed by its negative regulators including AMPK, phosphodiesterase (PDE) and protein phosphatase.[13,14] We then examined whether FFA inhibited CFTR channel activity via mechanisms involving activation of these enzymes. As shown in Fig. 3A, the inhibitory potency of FFA against forskolin-induced apical I_{Cl^-} was not affected by pretreatment with

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