

Cellular Mechanism Underlying Formaldehyde-Stimulated Cl^- Secretion in Rat Airway Epithelium

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Abstract

Background: Recent studies suggest that formaldehyde (FA) could be synthesized endogeneously and transient receptor potential (TRP) channel might be the sensor of FA. However, the physiological significance is still unclear.

Methodology/Principal Findings: The present study investigated the FA induced epithelial Cl^- secretion by activation of TRPV-1 channel located in the nerve ending fiber. Exogenously applied FA induced an increase of I_{SC} in intact rat trachea tissue but not in the primary cultured epithelial cells. Western blot and immunofluorescence analysis identified TRPV-1 expression in rat tracheal nerve ending. Capsazepine (CAZ), a TRPV-1 specific antagonist significantly blocked the I_{SC} induced by FA. The TRPV-1 agonist capsaicin (Cap) induced an increase of I_{SC} , which was similar to the I_{SC} induced by FA. L-703606, an NK-1 specific inhibitor and propranolol, an adrenalin β receptor inhibitor significantly abolished the I_{SC} induced by FA or Cap. In the ion substitute analysis, FA could not induce I_{SC} in the absence of extracellular Cl^- . The I_{SC} induced by FA could be blocked by the non-specific Cl^- channel inhibitor DPC and the CFTR specific inhibitor CFTR_{i-172}, but not by the Ca^{2+} -activated Cl^- channel inhibitor DIDS. Furthermore, both forskolin, an agonist of adenylate cyclase (AC) and MDL-12330A, an antagonist of AC could block FA-induced I_{SC} .

Conclusion: Our results suggest that FA-induced epithelial I_{SC} response is mediated by nerve, involving the activation of TRPV-1 and release of adrenalin as well as substance P.

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Introduction

For a long time, formaldehyde has been considered as a strongly stimulating environmental pollutant. Prolonged exposure to formaldehyde can cause asthma [1], [2], upper respiratory tract inflammation [3], [4], and pneumonodema, etc. Furthermore, formaldehyde has strong carcinogenic effects on human [5]. In the past, formaldehyde was classified mostly as a compound from extraneous contamination [6]. But recent investigations showed that *in vivo* methylamine can be catalyzed into formaldehyde by semicarbazide-sensitive amine oxidase [7], [8]. The physiological function of this endogenous formaldehyde is still unclear. Studies have demonstrated that endogenous formaldehyde could be related to inflammation responses [9].

TRP (transient receptor potential) channels are a group of non-selective cation channels that consist of six trans-membrane domains. TRP channels can be activated by a variety of factors such as temperature, ligands, mechanical force, pH, etc [10], [11]. They participate in related physiological responses as receptors for mechanical stimuli and other stimulation factors. As a member of the TRP family, TRPV-1 is expressed mostly in the terminals of sensory neurons and it is a mediator sensible to noxious thermal

and chemical agents [12], [13]. Studies have demonstrated that TRPV-1 plays an important role in the epidemic inflammation stimulated by formaldehyde [14]. Moreover, the endogenous formaldehyde of tumor tissues can activate TRPV-1 in acidic environment and induce bone cancer pain reactions [15]. Therefore, we hypothesized that formaldehyde might activate TRPV-1 in tracheal nerve endings and trigger a subsequent signal pathway in tracheal epithelium. In order to investigate this idea, we identified TRPV-1 expression in trachea and then we measured I_{SC} response in tracheal epithelium in the absence and presence of a variety of pharmacological modulators. According to our results, we propose a model for nerve-dependent regulation of epithelium Cl^- secretion in response to formaldehyde stimulation in rat trachea.

Results

FA-induced I_{SC} Response

The basal I_{SC} in isolated trachea was $8.22 \pm 1.64 \mu\text{A}/\text{cm}^2$ ($n = 28$) and application of FA (200 μM) to the basolateral side caused a sustained increase in I_{SC} (Fig. 1A). In addition, FA-induced I_{SC} was concentration-dependent (Fig. 1B) with an

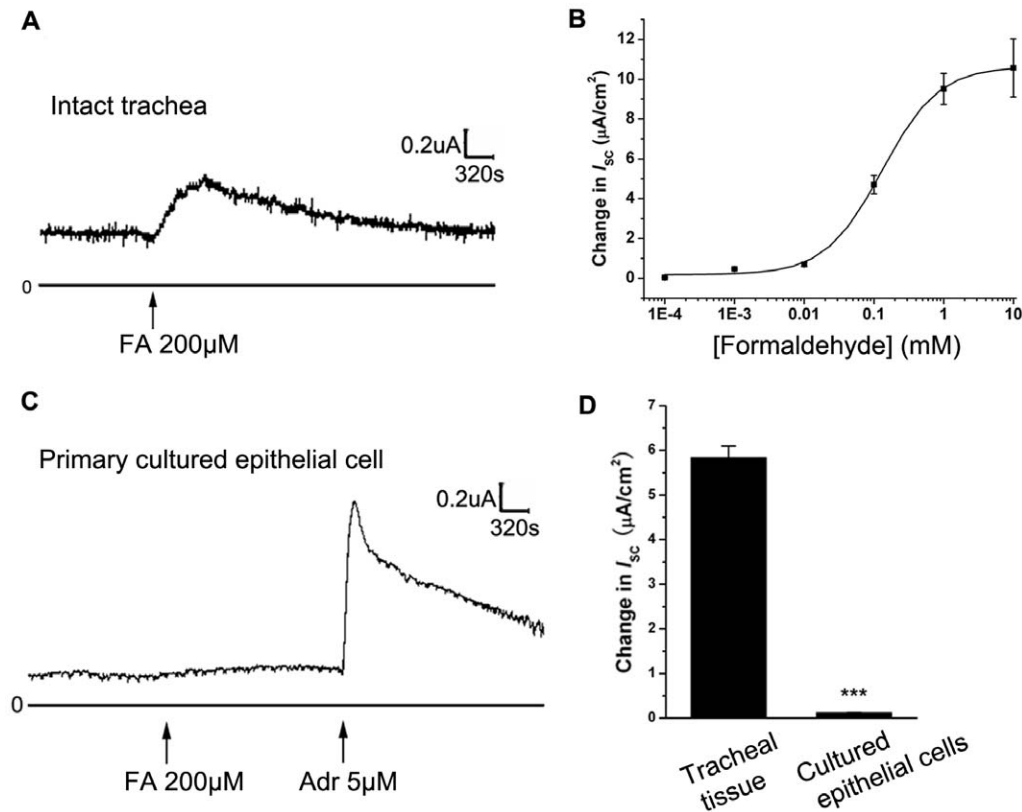


Figure 1. Effect of FA on I_{sc} in rat tracheal epithelium. (A) FA 200 μM applied to the basolateral side resulted in an increase in I_{sc} in intact tracheal tissue. (B) FA (0.1 μM -10 mM) stimulated a concentration-dependent I_{sc} response ($n=5$). Each data point represents a mean \pm SEM ($n=3\sim6$). (C) FA (200 μM) applied to the basolateral side resulted no increase in I_{sc} in cultured tracheal epithelial cells. ADR (5 μM) was added to demonstrate the good activity of epithelial cells. (D) Comparison of the peak magnitude of I_{sc} induced by FA in tracheal tissue and cultured cells. Values are mean \pm SEM ($n=4$, *** $p<0.001$). doi:10.1371/journal.pone.0054494.g001

apparent EC_{50} of about 0.13 ± 0.01 mM. Interestingly, in the primary cultured epithelial cells, FA (200 μM) could not induce an increase in I_{sc} (Fig. 1C and D), implying that I_{sc} induced by FA was nerve-mediated.

Expression and Localization of TRPV-1 in Rat Trachea

As demonstrated by western blot studies, TRPV-1 was expressed as proteins (Fig. 2). Immunofluorescence was used to clarify the location of TRPV-1. Double labeling TRPV-1 and Neurofilament-H (NF-H), a marker of nerve fibers in trachea showed that the locations of TRPV-1 and NF-H were mostly overlapped (Fig. 3A to F), suggesting that TRPV-1 was largely expressed in the intraepithelial nerve endings. Negative control was directly labeled with secondary antibodies without first antibodies (Fig. 3G, H and I).

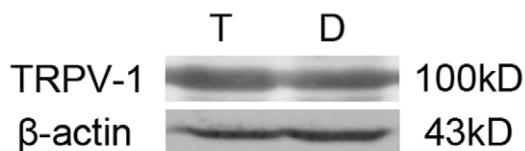


Figure 2. Representative western blot analysis for TRPV-1. T: tracheal tissue; D: dorsal root ganglion (DRG). β -actin served as loading control. doi:10.1371/journal.pone.0054494.g002

FA-induced I_{sc} was Mediated by TRPV-1 in Tracheal Nerve Ending

In order to investigate the involvement of TRPV-1 in tracheal nerve ending in the I_{sc} induced by FA, TRPV-1 specific antagonist and agonist were used. Pretreatment with TRPV-1 specific antagonist capsazepine (CAZ, 5 μM) to the basolateral side of the trachea significantly reduced the subsequent I_{sc} induced by FA (Fig. 4B). On the other hand, TRPV-1 agonist capsaicin (Cap, 5 μM) applied to the basolateral side could induce a sustained increase in I_{sc} (Fig. 4C), which was similar to the I_{sc} induced by FA (200 μM , Fig. 4A), and the Cap-induced I_{sc} could be completely abolished by CAZ (5 μM , Fig. 4D). These results clearly demonstrated that TRPV-1 in tracheal nerve ending was involved in the FA-induced I_{sc} .

Activation of TRPV-1 Induced the Release of Adrenalin and Activation of β -adrenergic Receptor in Epithelium

The nerve-dependent FA-induced I_{sc} might be mediated by the release of neurotransmitters from nerve. It has been reported that activation of TRPV-1 could increase the release of adrenalin [16]. Pretreated the trachea with propranolol (Prop, 10 μM), a β -adrenergic receptor inhibitor to the basolateral side, largely reduced the I_{sc} induced by FA (200 μM , Fig. 5A) or Cap (5 μM , Fig. 5B). But the basolateral application of phentolamine (Phen, 10 μM), an α -adrenergic receptor inhibitor, had no effect on the FA-induced I_{sc} (Fig. S1). These data indicate that activation of TRPV-1 by FA could release adrenalin from nerve

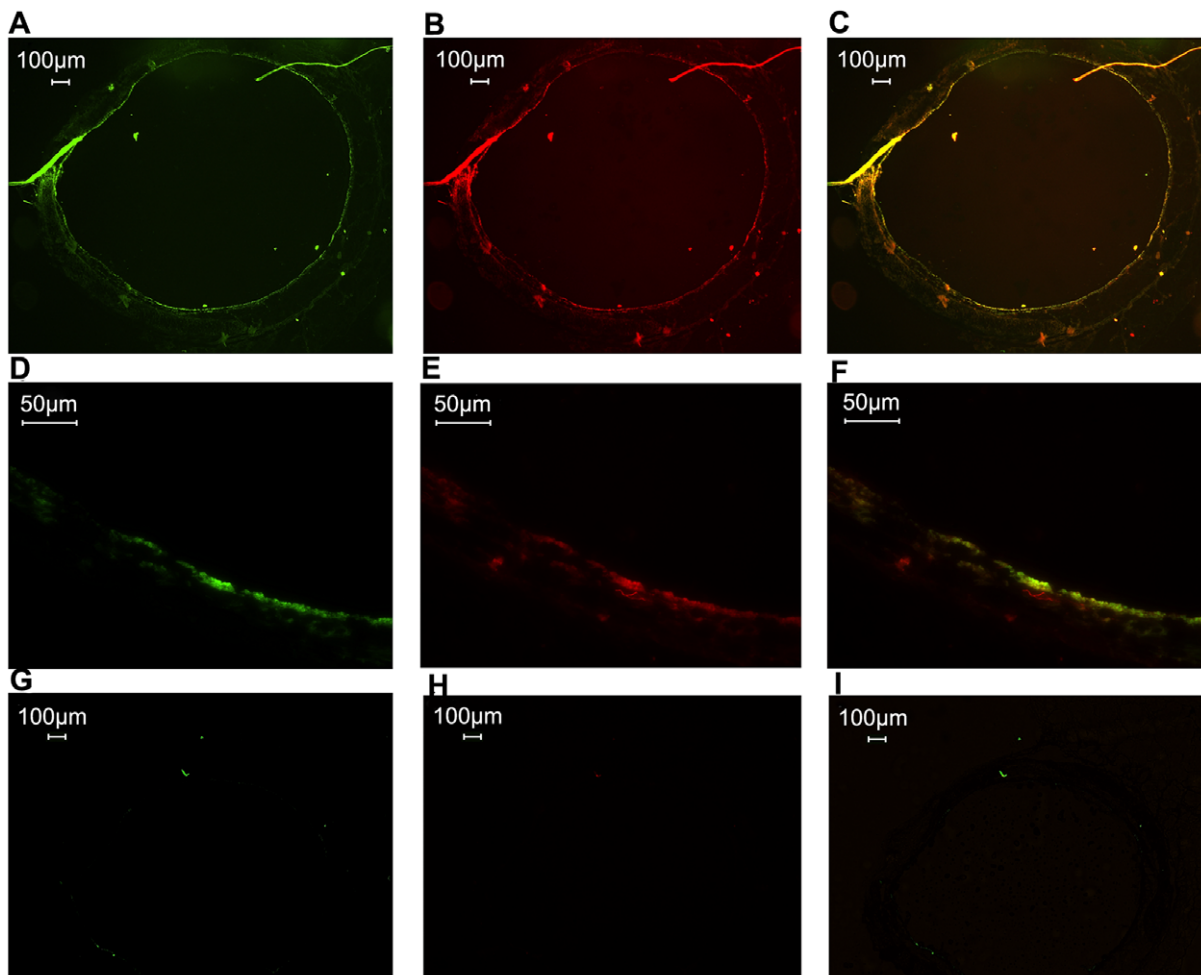


Figure 3. Double immunofluorescence localization of TRPV-1 and neurofilament in rat trachea. (A) and (D) fluorescence images of tracheal sections showing FITC immunoreactivity for TRPV-1. (B) and (E) fluorescence images of tracheal sections showing TR immunoreactivity for NF-H. (C) overlapping of (A) and (B). (F) overlapping of (D) and (E). (G) (H) and (I) negative control, no first antibody was used. doi:10.1371/journal.pone.0054494.g003

and activate the β -adrenergic receptor/ β -adrenergic receptor in epithelium.

Activation of TRPV-1 also Induced Release of Substance P and Activation of Neurokinin-1 Receptor in Epithelium

Since the β -adrenergic receptor inhibitor Prop could not completely abolish the I_{SC} response induced by FA or Cap, it is possible that some other neurotransmitters released from the nerve ending on account of activation of TRPV-1 may be involved in this process. It is well established that activation of TRPV-1 leads to release of substance P. To further test this idea, the neurokinin-1 receptor (NK-1R) specific inhibitor L-703606 (20 nM) was applied to the bathing solution of the basolateral side. The I_{SC} induced by FA (200 μM) or Cap (5 μM) was significantly reduced in the presence of L-703606 (Fig. 5E). Furthermore, after pretreatment with both Prop (10 μM) and L-703606 (20 nM), the I_{SC} induced by FA (200 μM , Fig. 5C) or Cap (5 μM , Fig. 5D) was almost completely blocked. These results suggest that activation of TRPV-1 may also lead to release of substance P and activation of NK-1R.

FA-induced I_{SC} Response was Mainly Cl^- Secretion

In order to study the ion species involved in the FA-induced I_{SC} , a series of ion substitution experiments were conducted. When trachea was bathed in normal K-H solution, FA induced a sustained increase in I_{SC} (Fig. 6A). Removing ambient HCO_3^- (HEPES buffering) had little effect on the FA-induced I_{SC} response (Fig. 6B). However, removing ambient Cl^- (gluconate substitution) or all anionic constituents abolished the FA-induced I_{SC} response (Fig. 6C and D). Summary of these effects is shown in Fig. 6E. In addition, application of the epithelial sodium channel (ENaC) inhibitor amiloride (100 μM) to the mucosal side had no effect on the FA-induced I_{SC} (Fig. S2), excluding the involvement of Na^+ reabsorption. All these results suggest that the FA-induced I_{SC} is primarily a Cl^- current.

Involvement of cAMP and CFTR in Mediating the Effect of FA

To further figure out which chloride channel participated in FA-induced I_{SC} response, different chloride channel inhibitors were applied. Either the non-selective chloride channel inhibitor DPC (1 mM, Fig. 7A) or the CFTR specific inhibitor CFTR_{i-172} (10 μM , Fig. 7B and C) applied to the mucosal side significantly blocked the I_{SC} induced by FA. But the Ca^{2+} activated chloride

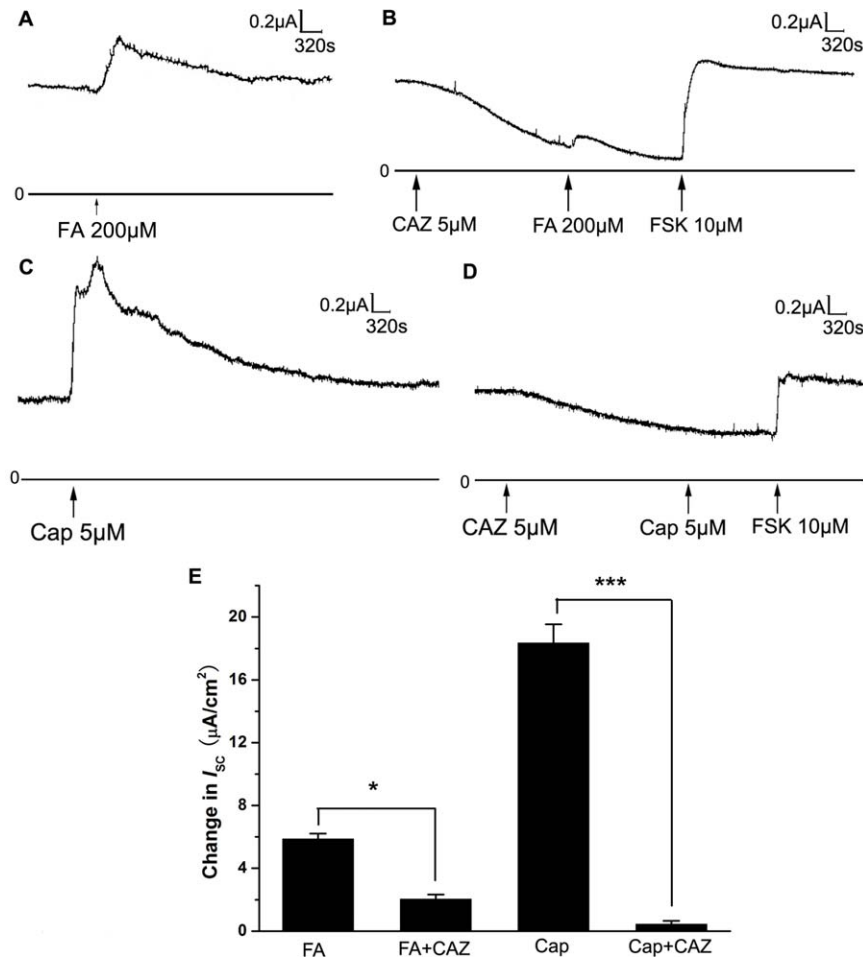


Figure 4. Effects of TRPV-1 agonist and antagonist on I_{sc} induced by FA. (A) I_{sc} response induced by FA (200 μM). (B) Representative mechanograms show the inhibitory effect of exogenously applied TRPV-1 antagonist CAZ (5 μM) on I_{sc} induced by FA (200 μM). (C) Representative mechanograms show the effect of TRPV-1 agonist Cap (5 μM) applied to the basolateral side on I_{sc} . (D) Representative mechanograms show the inhibitory effect of CAZ (5 μM) applied to the basolateral side on I_{sc} induced by Cap (5 μM). FSK (10 μM) applied to the basolateral side was added to demonstrate the good activity of tracheal tissue. (E) Summary results showing the effects of TRPV-1 agonist and antagonist on I_{sc} induced by FA (n=5, mean \pm SEM, * p<0.05, *** p<0.001). doi:10.1371/journal.pone.0054494.g004

channel (CaCC) inhibitor DIDS (100 μM) applied to the mucosal side had little effect on FA-induced I_{sc} (Fig. 7C). Fig. 7D showed the statistical analysis and the comparison of results between different chloride channel inhibitors on the FA-induced I_{sc} , revealing that the FA-induced Cl⁻ current is mediated by CFTR.

Nevertheless, how activation of β -adrenergic receptor could open CFTR and induce Cl⁻ secretion? One possibility is that activation of β -adrenergic receptor might increase the intracellular cAMP level [17] and thus activated CFTR. In order to test this hypothesis, AC agonist and antagonist were applied to the bathing solution. In the presence of forskolin (FSK, 10 μM , an AC activator), which was used to exhaust the AC so that there would be no further elevation in intracellular cAMP, the FA induced I_{sc} increase was almost completely abolished (Fig. 8A). Coordinately, pretreatment with MDL-12330A (10 μM , an inhibitor of AC) to the basolateral side of the trachea dramatically reduced the FA-induced I_{sc} compared with that in control group (Fig. 8B and C). These results confirm that the FA-induced I_{sc} is cAMP dependent.

Discussion

Formaldehyde had historically been known as a toxic gaseous molecule in the environment. Recent evidences have indicated that FA exists in most cells in human and other animals [18]. The physiological FA level was reported to be approximately 0.1 mM in the blood or brain of human and non-human animals [19]. Interestingly, some evidences have shown that endogenous FA concentration was significantly increased from the patients with tumor [20-22]. FA level was also elevated in lymphocytes in chronic lymphocytic leukemia [23]. However, the physiological function as well as the detailed signal pathway involving FA is still unknown yet.

Our present study has demonstrated for the first time that FA could induce a sustained increase in I_{sc} in the basolateral aspect of rat intact trachea (Fig. 1A). In addition, applying FA to the mucosal side has a similar effect as applying it to the basolateral side (data not shown). We suppose that this is because as a small-molecule gas, FA can go across the cells swiftly and induce the Cl⁻ currents. Interestingly, in primary cultured rat tracheal epithelial cells, FA could not induce the I_{sc} response (Fig. 1C). Since there were no nerve cells in cultured epithelium, there might be some

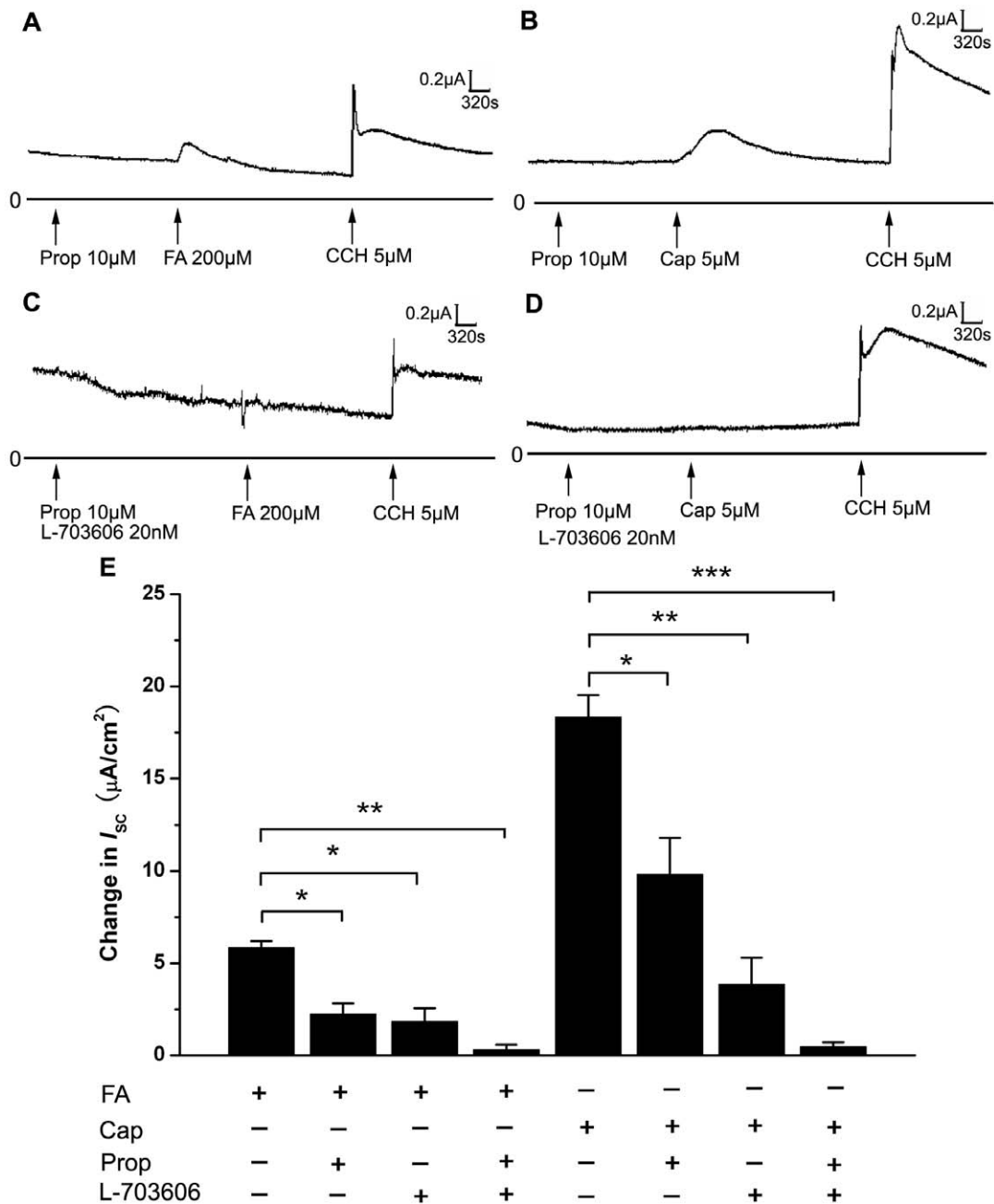


Figure 5. Effect of β -adrenergic receptor and neurokinin-1 receptor on I_{sc} induced by FA or Cap. Representative mechanograms show the inhibitory effects of exogenously applied the adrenalin β -receptor inhibitor propranolol (Prop, 10 μ M) to the basolateral side on I_{sc} induced by (A) FA (200 μ M) or (B) Cap (5 μ M) and exogenously applied Prop (10 μ M) as well as the neurokinin-1 (NK-1) specific inhibitor L-703606 (20 nM) to the basolateral side on I_{sc} induced by (C) FA (200 μ M) or (D) Cap (5 μ M). CCH (5 μ M) was added to the basolateral side to demonstrate the good activity of tracheal tissue. (E) Summary results showing the effects of Prop (10 μ M) and L-703606 (20 nM) on I_{sc} induced by FA (200 μ M, $n=5$) or Cap (5 μ M, $n=4$, mean \pm SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). doi:10.1371/journal.pone.0054494.g005

sensors in the nerve ending that participated in the reception of FA and caused the subsequent I_{sc} response.

In order to find out the sensor of FA in the nerve, TRPV-1 specific agonist and antagonist were used since previous evidence had suggested that TRP channel might be involved in the sensation of FA. In our experiments, we demonstrated that TRPV-1 was expressed and located in the nerve ending of rat trachea (Fig. 2 and 3). Furthermore, the TRPV-1 specific inhibitor CAZ could significantly block the I_{sc} induced by FA (Fig. 4B) and the

TRPV-1 agonist Cap could induce a similar I_{sc} response as FA did (Fig. 4C). These results indicated that TRPV-1 was involved in the I_{sc} induced by FA. Some recent studies also indicated that FA could induce TRPV-1 dependent current in DRG neurons and CHO cells expressing TRPV-1. [15], [24]. However, another group of researchers found that formalin could directly activate TRPA-1 in HEK-293 transfected with hTRPA1 and rTRPA1 respectively [25]. Since in our results CAZ could not completely abolish the I_{sc} induced by FA (Fig. 4B and E), TRPA-1 may also

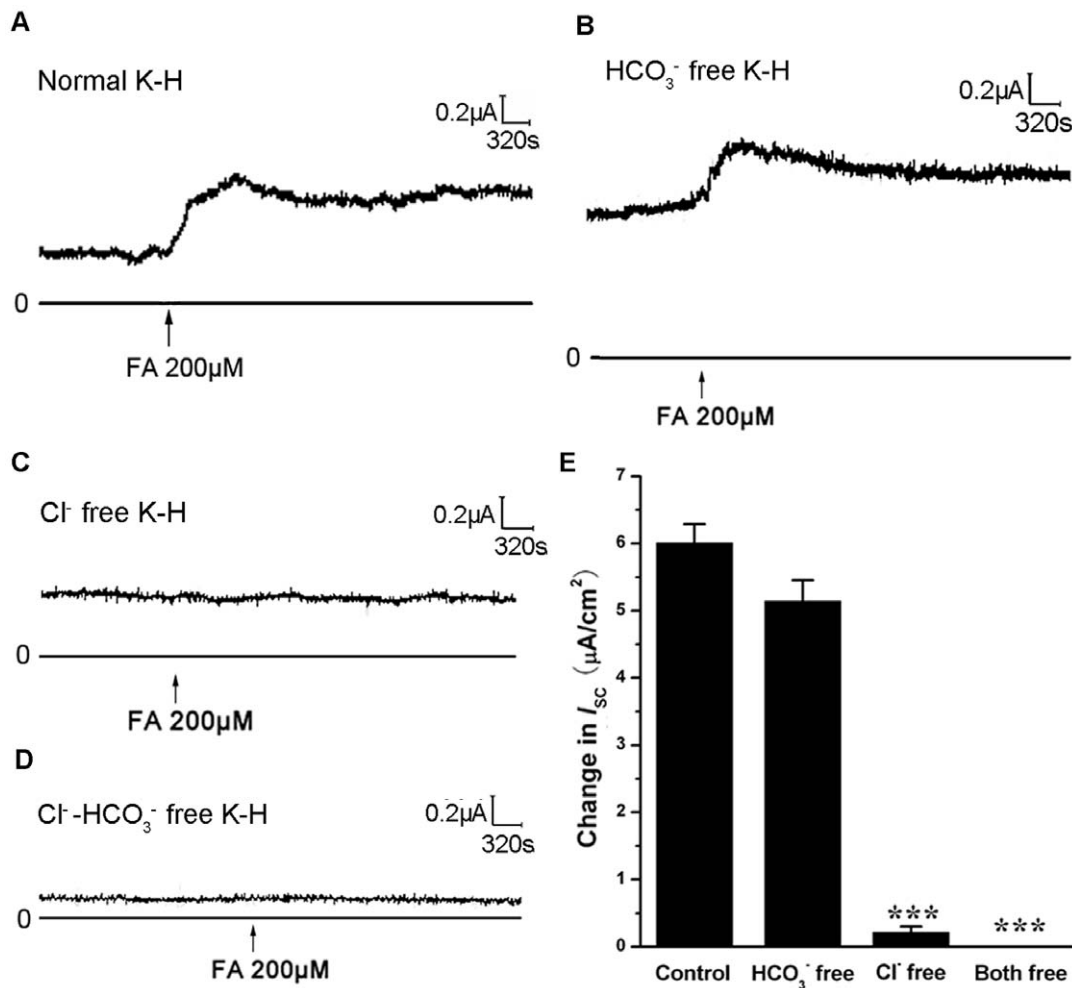


Figure 6. Replacement of extracellular anion resulted in different I_{sc} induced by FA. Representative I_{sc} recordings with arrows indicating FA (200 μM) were added in different K-H solutions. (A) Normal K-H (B) HCO_3^- free K-H (C) Cl^- free K-H and (D) Cl^- and HCO_3^- both free K-H. (E) Comparison of the peak magnitude of I_{sc} in different bathing solutions induced by FA(200 μM). Values are mean \pm SEM ($n=5$, *** $p<0.001$ vs. control).

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be involved in the sensation of FA. One possibility is that at low concentration FA may activate TRPA-1 while at high concentration FA activates both TRPA-1 and TRPV-1. Whether FA at 200 μM could activate TRPA-1 is still controversial and our results supported that FA at 200 μM could activate TRPV-1 in rat trachea.

It is well established that activation of TRPV-1 would induce the calcium influx and secrete a variety of neurotransmitters [26], [27]. Previous evidences also indicated that SP was released in the activation of TRPV-1 [28-30]. Our results further confirmed that activation of TRPV-1 would release SP since the NK1R inhibitor could partly block the I_{sc} induced by FA or Cap (Fig. 5E). In addition, the AdrR inhibitor Prop also partly abolished the I_{sc} response (Fig. 5A, B and E) and pretreatment with the NK1R inhibitor as well as Prop could almost completely block the I_{sc} response induced by FA or Cap (Fig. 5C, D and E), implying that not only SP but also adrenalin would be released from nerve endings in activation of TRPV-1.

How does the release of SP and Adr result in the I_{sc} response in epithelium? In previous studies, adrenalin could stimulate cAMP-dependent anion and fluid secretion through beta-adrenalin receptor activation [31-34]. In addition, SP could stimulate a

CFTR-dependent fluid secretion by airway submucosal glands [35-37]. In our present study, by using I_{sc} measurement, we demonstrated that FA could induce Cl^- secretion by inducing the release of adrenalin and SP, for the reason that FA could not induce the I_{sc} response in the Cl^- free bathing solution (Fig. 6C and D). Furthermore, non specific chloride channel inhibitor DPC and CFTR specific inhibitor CFTR_{i-172} could significantly abolish the I_{sc} response whereas CaCC inhibitor DIDS could not (Fig. 7). These data confirm that FA-induced chloride secretion is mediated by CFTR. The AC agonist or antagonist could block FA-induced I_{sc} response (Fig. 8), further indicating that FA induces a cAMP-dependent CFTR-mediated chloride secretion.

Based on the present findings, we propose a working model for nerve-epithelium intercellular communication. FA works as an important stimulus and SP as well as Adr are nerve-derived factors that regulate the tracheal epithelium anion secretion (Fig. 9). FA activates TRPV-1 in tracheal nerve ending, causing a Ca^{2+} influx, and thus induces the release of SP and Adr in the nerve. SP activates NK1R and Adr activates β -AdrR in epithelium, evoking the increase of cAMP and finally activating CFTR to induce a Cl^- secretion in tracheal epithelium. Interestingly, in addition to their effects on epithelium, nerve-derived factors may also influence

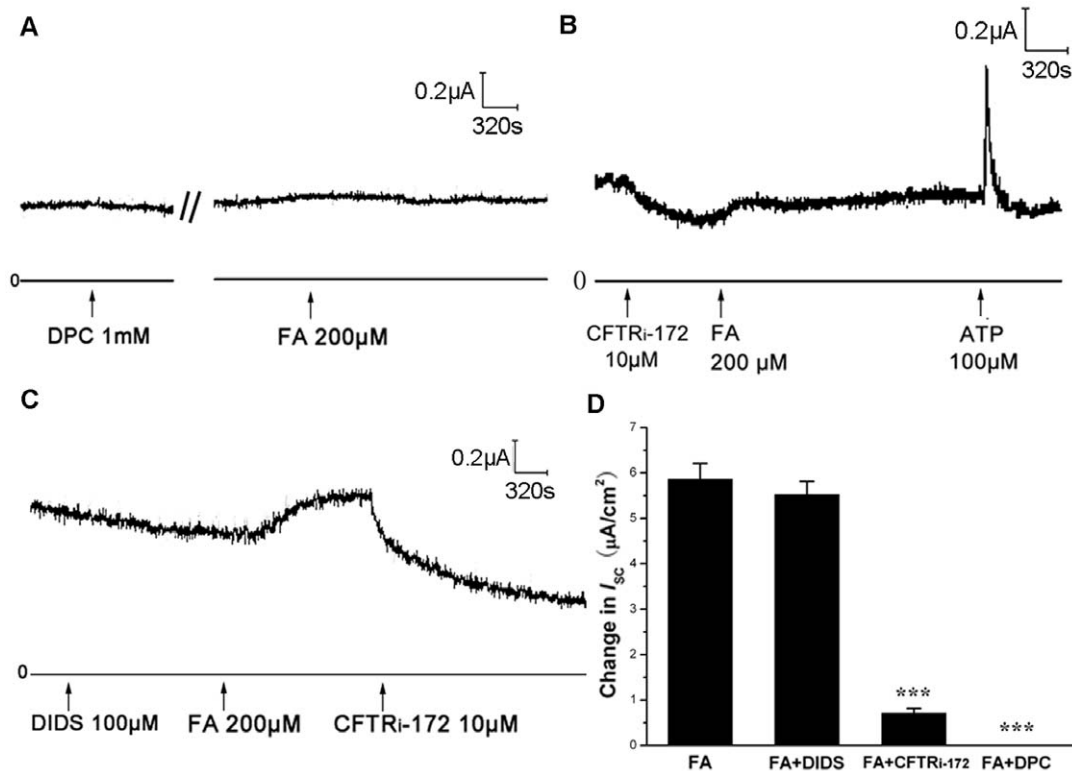


Figure 7. Effect of different chloride channel inhibitors on I_{sc} induced by FA. Representative traces showing the I_{sc} induced by FA (200 μM) pretreated with (A) the non-specific chloride channel inhibitor DPC (1 mM) to the mucosal side (B) the CFTR specific inhibitor CFTR_{i-172} (10 μM) to the mucosal side and (C) the Ca²⁺-activated chloride channel (CaCC) inhibitor DIDS (100 μM) to the mucosal side. (D) Summary results showing the effects of different chloride channel inhibitors on I_{sc} induced by FA (200 μM). Values are mean \pm SEM (n=6, ***p<0.001 vs. control). doi:10.1371/journal.pone.0054494.g007

smooth muscle cells or immune cells. For example, adrenalin could relax airway smooth muscles through the mediation of β 2-adrenoceptors [38]. Clinical studies also suggest that beta-receptor is an important target for the treatment of asthma and COPD [39–41]. In our experiments, FA-induced epithelium Cl⁻ secretion would further trigger the fluid transportation through epithelium. Therefore, in our model FA induces a nerve-derived release of adrenalin and SP leading to a CFTR-dependent chloride and fluid secretion, which might act as an innate defense response and would regulate the airway surface liquid volume in the extracellular environment. Our model also suggests an SP related inflammatory pathway at the high concentration of FA since SP is a well-known inflammatory factor. On the other hand, SP can activate NK1R in epithelium, evoking COX, which results in the generation of PGE₂ and activates EP_{2/4} receptors in airway smooth muscle, leading to an increase in cAMP [42]. This study provides a possible pathway in our model that PGE₂ may also activate EP_{2/4} receptors and increase the level of cAMP in epithelium to activate CFTR. In summary, the present study demonstrates that SP and Adr are important nerve-derived factors in FA-induced nerve-dependent CFTR-mediated Cl⁻ secretion. Thus, the present results provide a new insight into the physiological role of endogenous formaldehyde.

Materials and Methods

Ethics Statement

SPF Sprague-Dawley rats of 180 g–200 g were purchased from the Experimental Animal Center of Guangdong Province. Animals were housed in a constant-temperature (25°C) room

with a 12 h light–12 h dark photoperiod, according to the guidelines of the Sun Yat-sen University Animal Use Committee. All procedures were approved by the Sun Yat-sen University Animal Use Committee.

Measurement of I_{sc}

For all I_{sc} measurements, the main trachea of the rat airway system was cut into appropriate size and then mounted in hallowing paraffin between two halves of modified Ussing Chambers with an internal area of 0.03 cm². Electrodes for measuring transepithelial potential difference (PD) and passing current were connected to the chamber. The transepithelial PD was clamped at 0 mV, and then the short circuit currents were recorded with VCC MC6 voltage-current clamp amplifier (Physiologic instrument, San Diego, CA) and simultaneously displayed via a signal collection and analysis system (BL-420E⁺, Chengdu Technology & Market Co. Ltd, China). The change in I_{sc} was defined as the maximal rise in I_{sc} following agonist stimulation and was normalized to current change per unit area of the epithelial monolayer ($\mu A/cm^2$). The I_{sc} value was expressed as positive when the current flow from basolateral to apical [43].

Western Blotting

Total protein extract was obtained from rat trachea and dorsal root ganglion. The supernatant was collected from tissue homogenate after centrifugation. Equal amount of protein loaded in each lane was resolved by SDS-polyacrylamide gel electrophoresis and blotted on PVDF membrane. Membranes were blocked in Tris-buffered saline (TBS)/0.1% Tween-20 with 5% nonfat

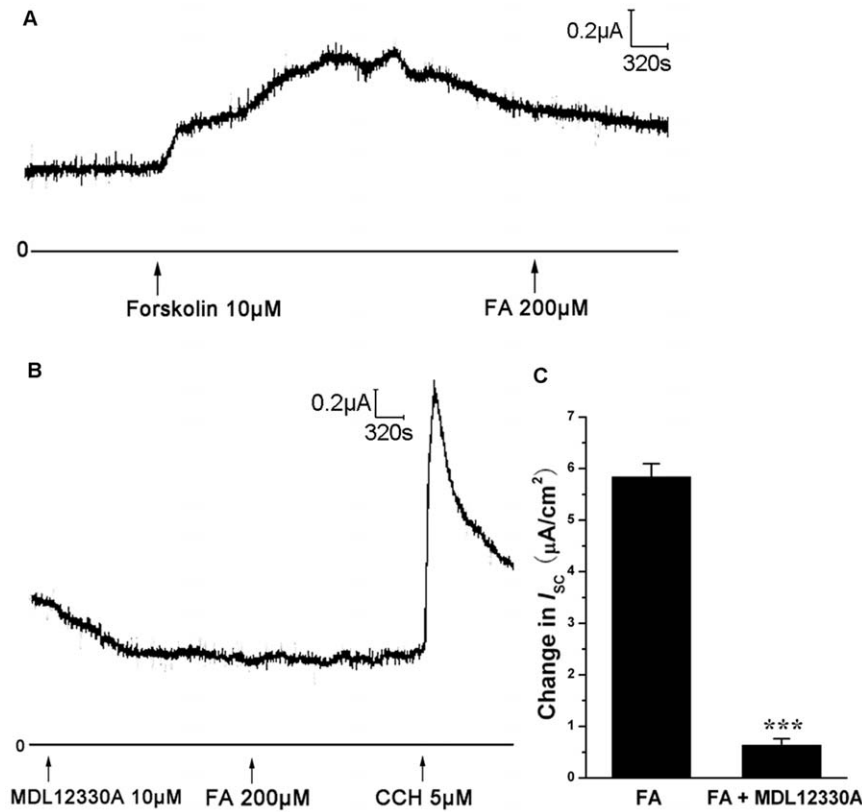


Figure 8. Effect of adenylate cyclase (AC) agonist or antagonist on I_{sc} induced by FA. (A) Pretreatment with the AC agonist forskolin (10 μ M) to the basolateral side abolished FA-induced I_{sc} . (B) Pretreatment with the AC antagonist MDL-12330A (10 μ M) to the basolateral side decreased FA-induced I_{sc} . CCH (5 μ M) was added to demonstrate the good activity of tracheal tissue. (C) Comparison of the peak magnitude of I_{sc} induced by FA with or without MDL-12330A. Values are mean \pm SEM (n=4, ***p<0.001). doi:10.1371/journal.pone.0054494.g008

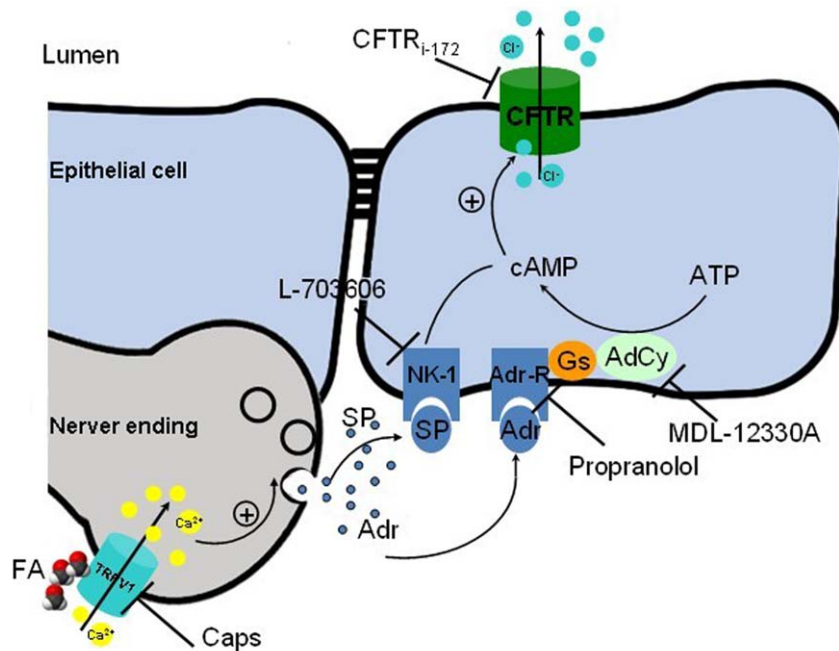


Figure 9. Cellular mechanism of formaldehyde caused Cl⁻ secretion in nerve-dependent epithelium. FA activates TRPV-1 in tracheal nerve ending, causing a Ca²⁺ influx, thus induces the release of SP and Adr in the nerve. SP activates NK1R and Adr activates β -AdrR in epithelium, evoking the increase of cAMP and finally activating CFTR to induce a Cl⁻ secretion in tracheal epithelium. doi:10.1371/journal.pone.0054494.g009

milk powder for 30 minutes at room temperature, and then incubated with specific antibody (Santa Cruz) for 2 h at room temperature. Membranes were incubated with peroxidase conjugated second antibody (Wuhan Boster Bio-engineering, Co. Ltd) for 1 h. The labeled proteins were detected with ECL kit.

Immunofluorescence

The main trachea of the rat airway system was removed rapidly and frozen in liquid nitrogen. Frozen tissues were sectioned by using a freezing microtome (Leica CM1850UV). For double staining, after the sections were pre-incubated with goat serum to minimize non-specific binding of IgG, they were incubated with first antibodies (rabbit TRPV-1, 1:200; mouse Neurofilament-H, NF-H, 1:200; Santa Cruz) at 4°C overnight. After washed for 3 times, sections were incubated with the appropriate secondary antibodies (anti-rabbit IgG-FITC, 1:100; anti-mouse IgG-TR, 1:100; Santa Cruz) for 1 h, then washed again in PBS, dehydrated in ethanol, air-dried, and mounted in Fluoromount. The specimens were examined under a fluorescence microscope (Leica DM5000B) at 546 nm and 490 nm wavelength.

Cell Culture

The procedures of tracheal epithelium primary culture have been described previously [44]. In brief, Sprague-Dawley rats weighing 180 g–200 g were killed by CO₂ inhalation. The main trachea was dissected out, finely minced with scissors, and treated successively with 0.25% (w/v) trypsin and 0.1% (w/v) collagenase. The disaggregated cells were suspended in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. After primary culture of 4–6 h, the cells were seeded onto Millipore filters (0.45 cm²) floating on DEME/F12 completed with other supplements. Cultured cells were incubated for 4 ~ 5 days at 37°C in 5% CO₂/95% O₂. Thereafter, the monolayers reached confluence and were ready for the measurement of I_{SC}.

Solutions and Chemicals

The normal KH solution contained (in mM) 117 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 2.56 CaCl₂, and 11.1 Glucose. The solution was kept at 37°C and gassed with 95% O₂ and 5% CO₂ to maintain the pH at 7.4. In Cl⁻ free Krebs solution, NaCl, KCl and CaCl₂ were replaced with the representative salts of gluconate, other compositions and condition

were not altered. In HCO₃⁻ free solution, HCO₃⁻ was substituted by 10 mM HEPES and the solution were bubbled with 100% O₂. In Cl⁻ and HCO₃⁻ free solution, the component is the same as Cl⁻ free solution, except NaHCO₃ was replaced with 20 mM Na-gluconate and 10 mM HEPES, and in the solution the gas was 100% O₂.

Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12), fetal bovine serum (FBS), penicillin/streptomycin, Hanks balanced salt solution and trypsin were purchased from Gibco Laboratories. DPC was bought from Riedel-de-Haen. Capsaicin, capsazepine, L-703606, propranolol, phentolamine, DIDS, CFTR_{i-172}, forskolin, MDL-12330A, CCH were purchased from Sigma Chemical Co. Rabbit TRPV-1, rabbit β-actin, mouse neurofilament-H, anti-rabbit IgG-FITC and anti-mouse IgG-TR were obtained from Santa Cruz biotechnology, INC.

Data Analysis and Statistics

Data were presented as Mean ± S.E.M. (n is the number of tissue preparations, or cells, or experiment times). For two groups of data, two-tail Student's t test was used. For more than three groups of data, ANOVA followed by Bonferroni's post hoc test was used. A value of P<0.05 was considered to be statistically significant.

Supporting Information

Figure S1 Effect of adrenalin α-receptor inhibitor on I_{SC} induced by FA. Representative mechanograms show the exogenously applied adrenalin α-receptor inhibitor phentolamine (Phen, 10 μM) to the basolateral side had no effect on I_{SC} induced by FA (200 μM) on tracheal tissue. (TIF)

Figure S2 Effect of sodium-hydrogen exchanger inhibitor on I_{SC} induced by FA. Representative mechanograms show the exogenously applied the epithelial sodium channel (ENaC) inhibitor amiloride (100 μM) to the mucosal side had no effect on I_{SC} induced by FA (200 μM) on tracheal tissue. (TIF)

Author Contributions

Conceived and designed the experiments: YLL JHH WLZ. Performed the experiments: YLL HMG YLZ. Analyzed the data: YLL HMG YXZ. Wrote the paper: YLL PXC JHH.

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