

EFFECTS OF T2R ACTIVATION ON DIFFERENT LUNG SENSORY SIGNALING

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## ABSTRACT

### EFFECTS OF T2R ACTIVATION ON DIFFERENT LUNG SENSORY SIGNALING

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Pharmaceuticals are commonly bitter tasting and therefore may activate bitter taste receptors (T2Rs), which are canonically expressed in the taste buds of the tongue. An accumulation of recent evidence has suggested T2Rs carrying out a diverse set of non-tasting functions that are critical to the maintenance of homeostasis in extraoral locations throughout the body which give rise to the possibility of new medications to target these receptors. Using whole-cell patch-clamp recordings, the present study aims to illustrate how the bitter tasting pharmaceuticals, through the activation of T2Rs, modulate the neuroplasticity and therefore the function of lung sensory neurons. Three different airway irritants were used to activate pulmonary sensory receptors: zinc, an agonist for TRPA1; low pH or acid, an activator for both acid-sensing ion channels (ASICs) and TRPV1; and ATP, an agonist for P2X purinoceptors. Our results show that pretreatment with T2Rs activator chloroquine (0.01, 0.1 and 1 mM, 90 s) concentration-dependently potentiates zinc (30  $\mu$ M, 3–16 s)-evoked TRPA1 currents and markedly inhibits ATP (0.3 or 1  $\mu$ M, 4–6 s)-evoked P2X currents, whereas affects acid (pH 5.5 and pH 6.5, 4–16 s)-evoked

inward currents differently: an inhibition for the slow inactivation ASIC-like current and a potentiation for the TRPV1-like current as well as the fast-inactivation ASIC current. Our results demonstrate that T2R activation in lung afferents has distinct modulatory effects on various ion channels that are sensitive to different airway irritants.

## INTRODUCTION

Gustatory perception is one of the five traditional senses and allows mammals to distinguish between sweet, sour, bitter and salty tastes (Chandrashekar et al., 2000). Of these differing perceptions, bitter taste in particular has the notable role of being a key defense mechanism against the ingestion of harmful substances, as many naturally occurring toxins are bitter tasting (Clark et al., 2012; Garcia & Hankins, 1975). Bitter taste receptors or T2Rs belong to a superfamily of seven-transmembrane G-protein-coupled receptors and are responsible for the perception of bitter taste (Lu et al., 2017). T2Rs were first identified in the oral cavity, but there has been a recent accumulation of evidence to support that these receptors are widely expressed throughout the body in the digestive, genitourinary, immune and respiratory systems (Ueda et al., 2003). T2Rs have been shown to carry out different specialized biological functions in these diverse locations where they are expressed (Ueda et al., 2003).

In the respiratory tract specifically, T2Rs have been reported to have various different functions to maintain airway homeostasis including: regulation of ciliary beating, production of inflammatory mediators and smooth muscle growth and tone (Finger et al., 2003; Krasteva et al., 2011; R. J. Lee et al., 2014; Shah et al., 2009). T2Rs' role in maintenance of homeostasis suggests that T2Rs are a possible target for new medicines and treatment of human disorders (Lu et al., 2017). In addition, understanding T2Rs may also help us to understand the side effects of bitter tasting medications that are currently being used today (Lu et al., 2017). Numerous studies have investigated the role of T2Rs in respiratory cells such as solitary chemosensory cells, ciliated respiratory epithelial cells, brush cells and airway smooth muscle, but the function of T2Rs in

pulmonary sensory neurons is still unknown (Shah et al., 2009; Sharma et al., 2015; Tizzano et al., 2010).

Vagal bronchopulmonary nerves are the primary communication pathway between the bronchopulmonary system and the central nervous system (Kollarik & Undem, 2004). Airway irritants that stimulate these airway afferents lead to a signal being transmitted to the central nervous system and integrated in the brainstem, which leads to various different airway reflexes such as reflex bronchoconstriction, cough, increased airway secretion and bronchial vasodilation (Kollarik & Undem, 2004). Non-myelinated C fibers account for more than seventy-five percent of the fibers in the pulmonary branch of the vagus nerve (Ho et al., 2001). These nociceptors can respond to thermal, mechanical and chemical stimuli, and are widely known for the expression of transient receptor potential vanilloid receptor 1 (TRPV1) (Craig, 2003). TRPV1 is partially responsible for C fiber polymodality as it is activated not only by vanilloid molecules including capsaicin, but also by protons, heat, anandamide and certain lipooxygenase metabolites of arachidonic acid (Everaerts et al., 2011; Pingle et al., 2007). Vagal bronchopulmonary nerves and the TRPV1 channel expressed in the majority of these nociceptors are critical to maintaining airway homeostasis and the manifestation of symptoms of airway hypersensitivity (Roberts et al., 1986).

We have recently reported that activation of T2Rs in lung nociceptors significantly augments capsaicin-evoked TRPV1 responses (Gu et al., 2017). We aim to further understand how T2R activation modulates the neuroplasticity and therefore the function of lung sensory neurons. In this study, we employ in-vitro whole-cell patch-clamping to investigate the integrated effects of T2R activation on various ion channels

that are sensitive to other airway irritants, including the transient receptor potential ankyrin 1 (TRPA1), acid-sensing ion channels (ASICs) and ATP-sensitive P2X receptors.

## MATERIALS AND METHODS

The procedures described were performed in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare) and US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training. These procedures were also approved by the Mercer University Institutional Animal Care and Use Committee.

*Labeling of vagal bronchopulmonary neurons.* Vagal bronchopulmonary sensory neurons were identified by retrograde labelling with the use of fluorescent tracer 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). Young Sprague-Dawley rats (80–120 g) were anesthetized via a nose-cone connected to a vaporizing machine with a continuous inhalation of isoflurane (Smiths Medical, Dublin, OH, USA). A small mid-line incision was made on the ventral neck skin to expose the trachea, and DiI (0.2 mg/ml, 50  $\mu$ l in volume) was instilled into the lungs via a 30-gauge needle inserted into the lumen of the trachea. The skin incision was then closed, and animals were kept undisturbed for 7–10 days until being euthanized and used for the cell culture.

*Isolation and culture of nodose and jugular ganglion neurons.* After being sufficiently anesthetized by isoflurane inhalation, rats were decapitated, and the head was immediately placed in ice-cold Dulbecco's modified Eagle's medium (DMEM)/F12 solution. The nodose and jugular ganglia were extracted and isolated under a dissecting microscope. Each ganglion was desheathed and cut into smaller pieces before being digested with 0.08% type IV collagenase for 60 min in an incubator. The suspension was centrifuged, and the supernatant was aspirated resulting in a cell pellet. The cell pellet was then resuspended and further digested in 0.05% trypsin for 1 min. The suspension

was again centrifuged, and the supernatant was aspirated. The cell pellet was resuspended in a modified DMEM/F12 solution [supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 100 µM minimum essential media non-essential amino acids] and gently triturated with a small-bore fire-polished Pasteur pipette. The isolated cells were plated onto poly-l-lysine-coated glass coverslips and incubated overnight (5% CO<sub>2</sub> in air at 37°C).

*Whole-cell perforated patch-clamp recordings.* Cultured cells were placed in a recording chamber with a steady flow of standard extracellular solution (ECS) containing (mM): 136 NaCl, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 0.33 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose and 10 HEPES; pH adjusted to 7.4 with NaOH. For solutions of pH 5.5, MES was used instead of HEPES for more reliable pH buffering. The intracellular solution (ICS) used in the micropipette contained (mM): 92 potassium gluconate, 40 KCl, 8 NaCl, 1 CaCl<sub>2</sub>, 0.5 MgCl<sub>2</sub>, 10 EGTA and 10 HEPES; pH adjusted to 7.2 with NaOH. Bronchopulmonary C fiber neurons were selected for by analyzing each cell for specific traits including: fluorescence intensity in UV indicating labelling by DiI, cell diameter <35 µm and response to 0.75 µM capsaicin. The resting membrane potential was held at -70 mV after micropipette-cell seal is achieved. The chemical stimulants were applied with a pressure-driven drug-delivery system (VC<sup>38</sup>; ALA Scientific Instruments, Westbury, NY, USA). All whole-cell perforated (50 µg/ml gramicidin) patch-clamp recordings were obtained using Axopatch 200B, Digidata 1440A and pCLAMP 10 software. Data from nodose and jugular ganglion neurons were pooled for group analysis since no difference was found between responses of the neurons obtained from these two ganglia.

*Chemicals.* All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). A stock solution of capsaicin 1 mM was prepared in 1% Tween 80, 1% ethanol and 98% saline. The stock solution of ZnCl<sub>2</sub> (1 M) was prepared in distilled water. Solutions of chemical agents at desired concentrations were prepared daily with ECS before experiments.

*Statistical analysis.* Data was analyzed with a one-way repeated-measure ANOVA. When the ANOVA showed a significant interaction, pairwise comparisons were made with Fisher's least significant difference post hoc analysis. A value of  $P < 0.05$  was considered to be significant. Data are reported as means  $\pm$  SEM.

## RESULTS

*T2R agonist chloroquine activated a subpopulation of pulmonary sensory neurons.* Incubation with 1, 10, 100 and 1000  $\mu\text{M}$  chloroquine for 90 s evoked inward currents in 13.3%, 41.9%, 51.6% and 68.1% of neurons, with an average amplitude of  $3.5 \pm 2.4$ ,  $10.2 \pm 3.3$ ,  $23.1 \pm 5.2$  and  $66.3 \pm 14.1$  pA, respectively (n = 20–64).

*Activation of T2Rs with chloroquine potentiated capsaicin-evoked TRPV1 currents in pulmonary nociceptors.* Transient application of capsaicin (0.3 or 1  $\mu\text{M}$ , 2–10 s) evoked a whole-cell inward current in 71.2% (47 out of 66) pulmonary sensory neurons which are known to be TRPV1 expressing nociceptors (Lee & Gu, 2009). Pretreatment with chloroquine for 90 s concentration-dependently potentiated capsaicin-evoked TRPV1 currents in all capsaicin-sensitive neurons tested (Fig. 1). The amplitude of capsaicin currents was significantly increased from  $346.6 \pm 46.5$  at control to  $518.9 \pm 90.4$ ,  $727.4 \pm 129.0$  and  $1282.3 \pm 274.2$  pA (n = 13, P < 0.05) after 90 s pretreatment with 10, 100 and 1000  $\mu\text{M}$  chloroquine, respectively. The enhanced capsaicin current returned towards control level when tested again after 10 min wash.

*Pretreatment with chloroquine also enhanced ZnCl<sub>2</sub>-evoked TRPA1 currents.* As reported recently from our lab, application of heavy metal zinc evokes an inward current in a subset of capsaicin-sensitive pulmonary sensory neurons that is mediated through the activation of TRPA1 channels, as evidenced by its inhibition by AP18, a specific TRPA1 antagonist (Gu & Lin 2010 ; Vysotskaya et al. 2014). As shown in Fig. 2, pretreatment with chloroquine concentration-dependently enhanced the inward currents evoked by ZnCl<sub>2</sub> application in pulmonary sensory neurons. ZnCl<sub>2</sub> (30  $\mu\text{M}$ , 3–16 s)-evoked TRPA1 currents were increased to  $151.0 \pm 10.3$  %,  $208.8 \pm 14.4$  % and  $318.7 \pm 43.9$  % (n =

10–18,  $P < 0.05$ ) of their control responses after 90-s pretreatment with 0.01, 0.1 and 1 mM chloroquine, respectively. The sensitizing effect on ZnCl<sub>2</sub> current was reversible within 10 min wash after the termination of chloroquine treatment.

*Pretreatment with chloroquine inhibited ATP-evoked inward currents.* ATP is known to induce an inward current via the activation of P2X channels in peripheral neurons including pulmonary sensory neurons (Taylor-Clark & Udem, 2006 ). Unlike the sensitizing effect on capsaicin-evoked TRPV1 and zinc-evoked TRPA1 currents, pretreatment of 1 mM chloroquine for 90 s significantly inhibited the ATP (1  $\mu$ M, 4 s)-induced P2X current to  $52.6 \pm 9.7$  % of its control amplitude ( $n = 11$ ,  $P < 0.05$ ). The ATP-evoked current was also reduced after pretreatment with lower concentrations of chloroquine (0.01 and 0.1 mM, 90 s), but the reduction was not significant when compared with its corresponding control ( $n = 5$  and  $10$ ,  $P > 0.05$ ) (Fig. 3).

*Effects of chloroquine pretreatment on acid-induced ASIC- and TRPV1-like currents in pulmonary sensory neurons.* Local tissue acidosis can occur in various physiological and pathophysiological states, including inflammation, infection, ischemia, cancer, tissue injury and metabolic stress (Gu & Lee, 2010 ). Acid is known to directly activate two groups of ion channels in pulmonary sensory neurons: ASICs and TRPV1 (Gu & Lee, 2006 ). A pH drop from 7.4 to 6.5 was used to evoke an ASIC-like current and a low pH of 5.5 was used to elicit a TRPV1-like current; specific antagonists for ASICs and TRPV1 were not employed in this study. Interestingly, our data showed that pretreatment with T2Rs activator chloroquine (0.01, 0.1 and 1 mM, 90 s) affected acid-evoked inward currents differently. It inhibited pH 6.5-evoked slow inactivation ASIC-

like current (Fig. 4), whereas potentiated pH 5.5-evoked TRPV1-like current (Fig. 5) as well as the fast-inactivation ASIC current (Fig. 6).

## DISCUSSION

The expression and function of extraoral T2Rs has been an emerging area of interest with the majority of recent studies focusing on T2Rs' role in innate immunity in the respiratory system (Chandrashekar et al., 2000). The evidence thus far has shown T2R-mediated signaling greatly contributes to the innate immunity in the epithelia of the organs that are connected to the external environment (Lu et al., 2017). In the mouse nasal cavity, activation of T2Rs in solitary chemosensory cells stimulated trigeminal fibers resulting in the initiation of a neurogenic inflammation response to block bacterial invasion (Tizzano et al., 2010). In human sinonasal epithelia, activation of T2Rs in solitary chemosensory cells resulted in increases of calcium ions in surrounding gap junctions causing secretions of antimicrobial peptides and  $\beta$ -defensin (R. J. Lee et al., 2014). A function for the brush cells located in the respiratory system has not yet been attributed with certainty, but there is evidence to suggest that they act as immune surveillance against air pollutants and infectious agents (Reid et al., 2005). In the mouse trachea, activation of brush cells expressing T2Rs lead to regulation of respiratory rates (Finger et al., 2003; Krasteva et al., 2011). Furthermore, ciliated human epithelial airway cells express multiple T2R subtypes with activation leading to concentration-dependent increases in intracellular calcium ion concentration (Shah et al., 2009). The increases in calcium ion concentration resulted in increases in ciliary beat frequency aiding the elimination of noxious substances from the airway (Shah et al., 2009). The activation of T2Rs in airway smooth muscles cells by bitter tastants also followed a concentration-dependent response resulting in relaxing of precontracted airway muscle (Deshpande et al., 2010).

There are approximately thirty different T2R receptor subtypes that have been found in humans and over a hundred natural and synthetic bitter tasting compounds which have been shown to activate T2Rs (Andres-Barquin & Conte, 2004). The known agonists of T2Rs activate multiple subtypes, and selective agonists and antagonists for specific T2R subtypes are not yet available (Jaggupilli, Howard, Upadhyaya, Bhullar, & Chelikani, 2016). With the use of patch-clamp recordings, we tested the effects of three commonly used T2R agonists with the same concentration and condition on the capsaicin-evoked TRPV1 current (Gu et al., 2017). All three of the agonists significantly potentiated the capsaicin-evoked current with chloroquine being the most potent followed by diphenitol and lastly denatonium. We were unable to determine the T2R receptor subtypes that were primarily involved due to the lack of specific T2R subtype antagonists, however the potentiation of capsaicin-evoked currents is likely mediated through T2Rs and not a non-specific toxic effect because: chloroquine has a clear concentration-dependent effect on the capsaicin-evoked current; the effects of the structurally dissimilar compounds chloroquine, diphenitol, and denatonium had similar effects; and the potentiation in the patch-clamp recording returned to control levels after cessation of chloroquine treatment.

TRPV1 is a non-selective cation channel known for its role as a molecular integrator of inflammatory mediators (Szallasi et al., 2007). The TRPV1 channel responds to multiple physiological and environmental stimuli such as capsaicin, temperature, protons and lipoxygenase metabolites, and has been shown to be sensitized by a wide range of agents (Everaerts et al., 2011). The activation of TRPV1-expressing bronchopulmonary neurons leads to numerous different airway reflex responses

including: reflex bronchoconstriction, mucus hyper secretion, airway irritation, and the urge to cough (L.-Y. Lee & Gu, 2009). TRPA1 is yet another member of the TRP channel family that has attracted attention for its potential role in nociception and neurogenic inflammation. Similar to the TRPV1 channel, TRPA1 responds to a multitude of different ligands, some of which are: isocyanates, oxidants, ROS mediators, heavy metals, anesthetics and tobacco smoke (Bautista et al., 2006). Together with TRPV1, the capsaicin receptor, TRPA1 may contribute to chemical hypersensitivity, chronic cough, and airway inflammation in asthma, COPD and reactive airway dysfunction syndrome (Bessac & Jordt, 2008). In this study, our results show that T2R activation after chloroquine pretreatment concentration-dependently augment both capsaicin-evoked TRPV1 currents and ZnCl<sub>2</sub>-evoked TRPA1 currents. We have also studied the effects of T2R activation on channels present in vagal bronchopulmonary neurons that respond to extracellular protons and ATP. The release of ATP from airway epithelial cells during inflammatory process is considered to play an important role in the pathophysiology of asthma and chronic obstructive pulmonary disease, and the pH of deaerated exhaled airway vapor condensate is over two log orders lower in patients with acute asthma (Hunt et al., 2000; Oguma et al., 2007). Activation of T2Rs with chloroquine had an inhibitory effect on the P2X purinoreceptors and the acid sensing ion channels at pH 6.5, which respond to extracellular ATP and extracellular hydrogen respectively. However, pH 5.5-evoked TRPV1-like currents and fast-inactivation ASIC-like currents were both concentration dependently enhanced by the chloroquine pretreatment.

It is known that many clinical drugs have bitter taste, and these “bitter pills” suffer from low patient compliance due to aversion to their taste (Levit et al. 2014). In

addition, many bitter-tasting medications have other off-target side effects including those affecting respiratory system. For example, antiarrhythmic drug propafenone is known to cause dyspnea, wheezing, or bronchoconstriction, and these side effects have not exclusively been reported in patients with preexisting reactive airways disease (Hill et al. 1986 ; Olm et al. 1989). The physiological significance of our findings in this study is not yet clear. However, it is plausible to assume that many off-target respiratory effects of bitter tasting drugs could be resulted at least partly from activation of T2Rs in pulmonary sensory neurons and the subsequent cardiopulmonary reflexes.

In summary, our study demonstrates that T2R activation in lung afferents has distinct modulatory effects on various ion channels that are sensitive to different airway irritants. Our results provide direct support on the cellular and molecular level for the previous findings by Zervakis et al. (2000) that when tested in humans, bitter tasting drugs can increase the perceived intensity of some tastants while decreasing others, presumably the reason for the taste distortion and dysgeusia induced by some bitter pills. In future studies, we intend to identify which T2R subtypes that the bitter tasting drugs activate in the airway sensory neurons and investigate the transduction pathways underlying the distinct regulatory effects of T2Rs activation on different airway sensory receptors. Since activation of T2Rs appears to generate a local neurogenic inflammation in the lungs and airways, they could serve as potential targets for new drugs to treat airway diseases such as asthma.

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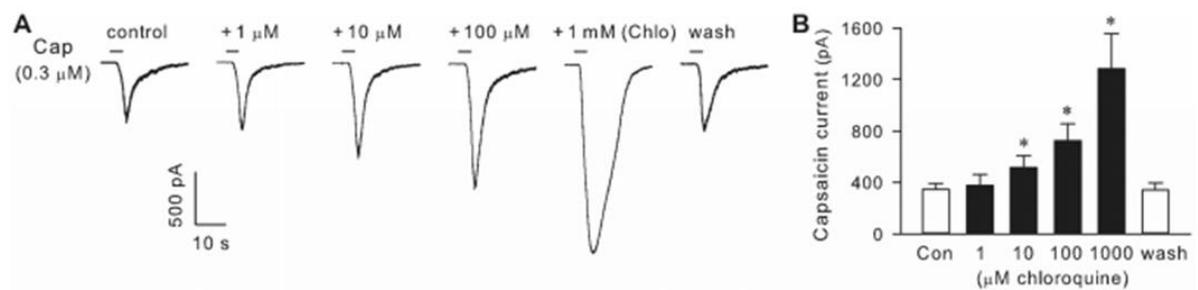
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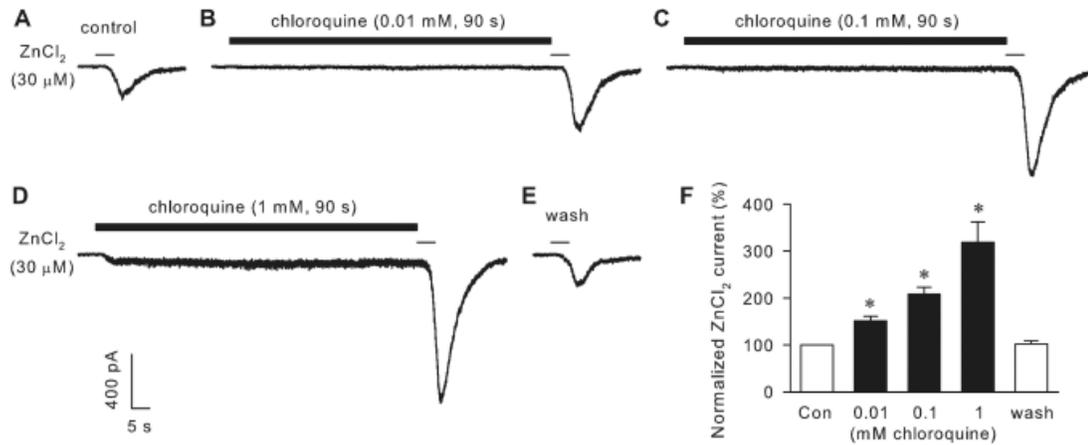
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APPENDIX

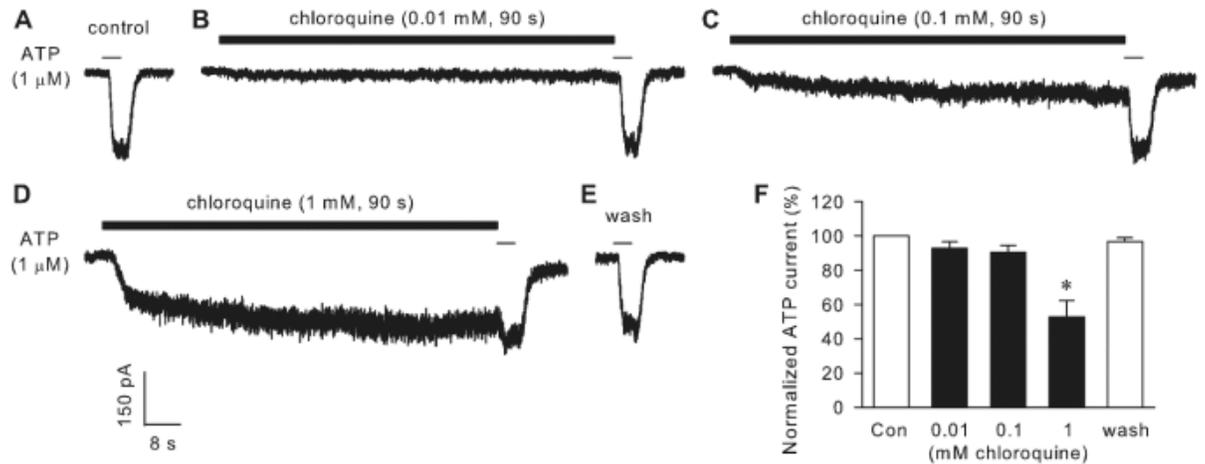
FIGURES



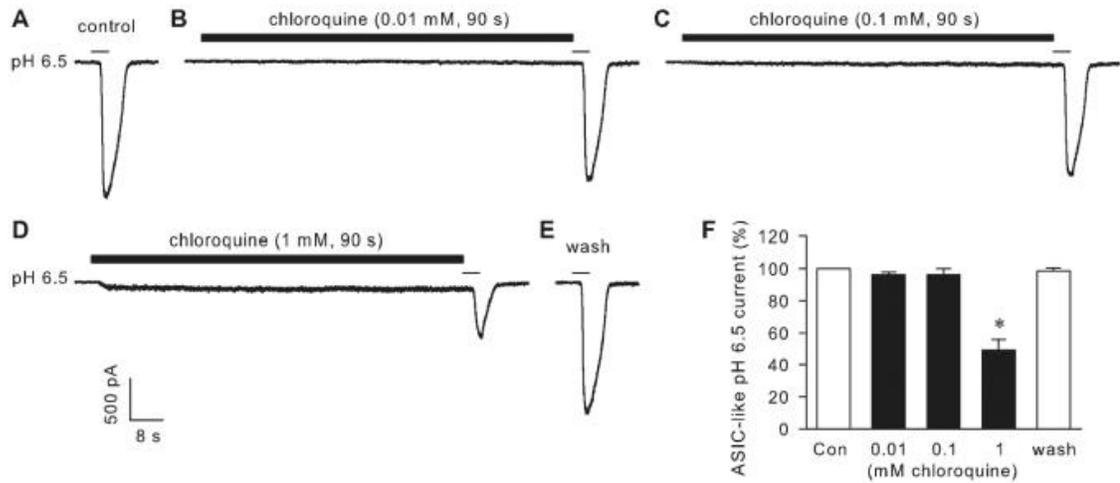
**Fig. 1. Pretreatment with T2Rs agonist chloroquine potentiates capsaicin-evoked currents in rat vagal bronchopulmonary sensory neurons.** (A) Inward currents evoked by capsaicin (Cap; 0.3 μM, 4 s) at control and after 90-s pretreatment with increasing concentrations of chloroquine (1 μM to 1 mM). (B) Group data showing that chloroquine concentration-dependently enhances capsaicin-evoked currents (n = 13, P < 0.05). The order of applications of different concentrations of chloroquine was randomized in different neurons. \*Significantly different from the control response.



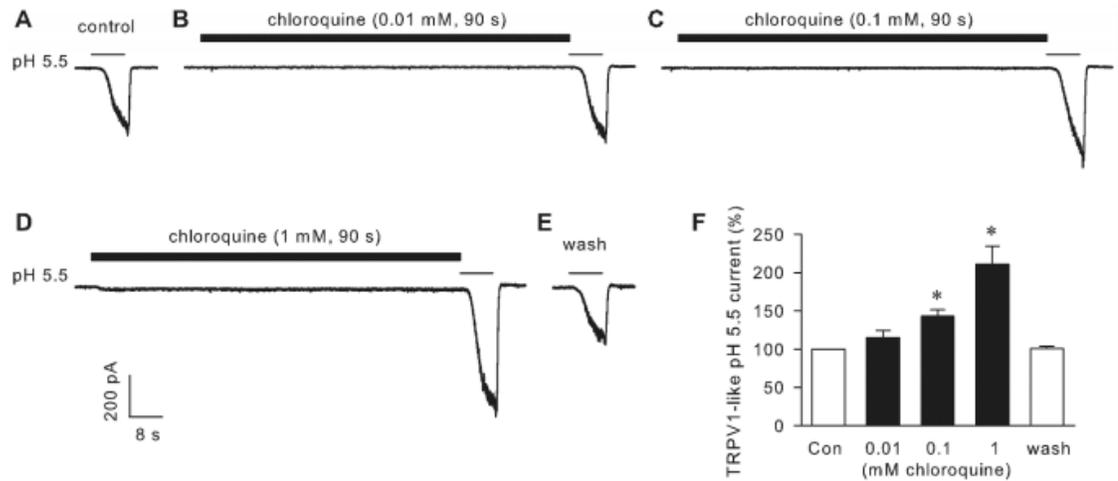
**Fig. 2. Pretreatment with chloroquine potentiates ZnCl<sub>2</sub>-evoked TRPA1 currents.** (A) Inward currents evoked by ZnCl<sub>2</sub> (30 μM, 5 s) at control and after 90-s pretreatment with increasing concentrations of chloroquine (0.01–1 mM). (B) Group data showing that chloroquine concentration-dependently enhances ZnCl<sub>2</sub>-evoked currents (n = 10–18, P < 0.05). \*Significantly different from the control response.



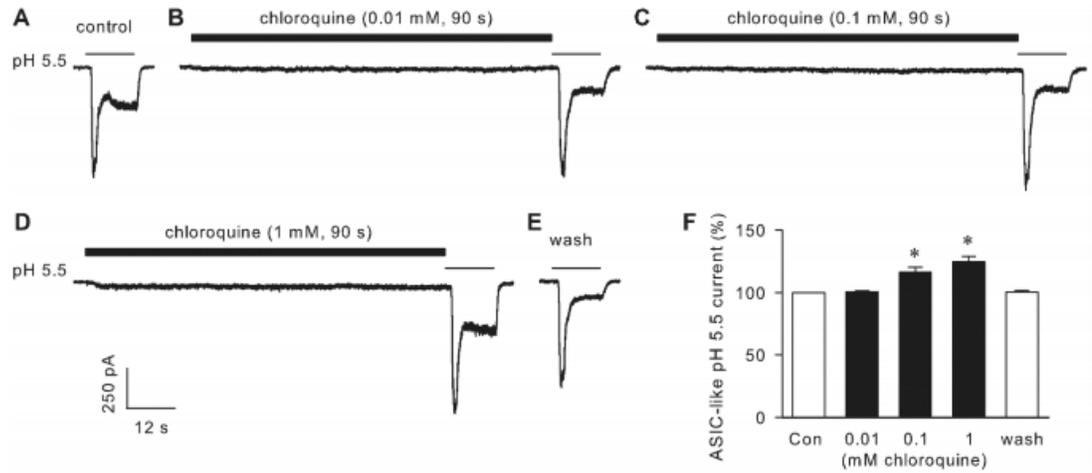
**Fig. 3. Pretreatment with chloroquine inhibits ATP-evoked P2X currents.** (A) Inward currents evoked by ATP (1  $\mu$ M, 4 s) at control and after 90-s pretreatment with increasing concentrations of chloroquine (0.01–1 mM). (B) Group data showing the inhibition of ATP currents by chloroquine pretreatment (n = 5–11, P < 0.05). \*Significantly different from the control response.



**Fig. 4. Pretreatment with chloroquine inhibits pH 6.5-evoked ASIC-like currents.** (A) Inward CONCLUSION currents evoked by pH 6.5 (4 s) at control and after 90-s pretreatment with increasing concentrations of chloroquine (0.01–1 mM). (B) Group data showing the inhibition of pH 6.5- evoked ASIC-like currents by chloroquine pretreatment (n = 15–18, P < 0.05). \*Significantly different from the control response.



**Fig. 5. Pretreatment with chloroquine enhances pH 5.5-evoked TRPV1-like currents.** (A) Inward currents evoked by pH 5.5 (8 s) at control and after 90-s pretreatment with increasing concentrations of chloroquine (0.01–1 mM). (B) Group data showing the potentiation of pH 5.5- evoked TRPV1-like currents by chloroquine pretreatment (n = 6–16, P < 0.05). \*Significantly different from the control response.



**Fig. 6. Pretreatment with chloroquine enhances pH 5.5-evoked fast inactivation ASIC-like currents.** (A) Inward currents evoked by pH 5.5 (12 s) at control and after 90-s pretreatment with increasing concentrations of chloroquine (0.01–1 mM). (B) Group data showing the potentiation of pH 5.5-evoked fast inactivation ASIC-like currents by chloroquine pretreatment (n = 5–8, P < 0.05). \*Significantly different from the control response.