Possible contribution of pannexin-1 to capsaicin-induced ATP release in rat nasal columnar epithelial cells

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ABSTRACT

Current evidence indicates that transient receptor potential (TRP) channel activity involves a relationship between opening of pannexin-1 and release of ATP into the extracellular space. We examined the effects of agonists of thermosensitive TRP channels (TRPM8, TRPA1, TRPV1, and TRPV2) on ATP release from rat nasal mucosa, and measured ciliary beat frequency (CBF) using digital high-speed video imaging. Single-cell patch clamping from dissociated rat nasal columnar epithelial cells was performed to confirm the relationship between pannexin-1 and TRP. We demonstrated that ATP release and CBF were significantly potentiated by the heat-sensitive TRPV1 agonist capsaicin (10 μM), but not by other TRP agonists. Capsaicin-induced ATP release and CBF increase were significantly inhibited by the pannexin-1 blockers carbenoxolone (10 μM) and probenecid (300 μM). In addition, the voltage step-evoked currents in the presence of capsaicin were inhibited by the pannexin-1 blockers in single-cell patch clamping. Our results suggest the participation of TRPV1 and pannexin-1 in the physiologic functions of rat nasal mucosa.

INTRODUCTION

Pannexins are a family of transmembrane channel proteins in vertebrates that are homologous to the invertebrate gap junction proteins known as innexins. None of the 3 subtypes of pannexins, pannexin-1, -2, or -3, have significant sequence similarity to connexins, which are the prototypical vertebrate gap junction proteins. Pannexin-1 is the most thoroughly investigated member of the pannexin family and forms an ATP-permeable, voltage-dependent large-conductance (approximately 500 pS), nonselective channel.

In the airway, extracellular ATP plays an important role in regulating mucus/ion secretion and mucociliary clearance. We previously showed, via immunohistochemical and molecular biologic studies, that pannexin-1 is expressed in the epithelial layer of rat nasal mucosa. Current evidence suggests that the opening of pannexin-1 and release of ATP into the extracellular space is related to the activity of several kinds of transient receptor potential (TRP) channels. The TRP family includes thermosensitive cation channels, such as the cold sensors TRPM8 and TRPA1 and the heat sensors TRPV1 and TRPV2. Because the temperature of the nasal mucosa fluctuates along with the breath, we hypothesized that pannexin-1 in the nasal mucosa likely plays a role in the potentiation of ATP release via thermosensors, such as through the activation of TRP channels.

In the present study, we investigated the interactions among ATP release, TRP channel activity, and pannexin-1 function in rat nasal mucosa using agonists specific to various TRP channels, alone and in combination. The effect of these treatments on ciliary beat frequency (CBF) was also examined. The results describe a role for the TRPV1 and pannexin-1 functional axis in the regulation of ciliary movement.

RESULTS

Time-course measurements of ATP release from rat nasal mucosa under various conditions are summarized in Fig. 1. After 5-min challenges with TRPM8...
agonist menthol (10 mM; Fig 1A), TRPA1 agonist cinnamaldehyde (10 mM; Fig 1B), and TRPV2 agonist cannabidiol (1 μM; Fig. 1C), ATP concentrations with addition of 10 μM capsaicin were 10.3 ± 2.0 fM and 8.25 ± 1.7 fM (n = 12) after 5-min and 10-min applications, respectively, significantly higher than the basal value of 2.17 ± 0.5 fM (*, p < 0.05 in both cases). In contrast, 10 mM menthol (n = 5), 10 mM cinnamaldehyde (n = 5), and 1 μM cannabidiol (n = 6) showed no significant effects on ATP release. The time points of −5 and 0 min represent the soaking of mucosal segments in 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer solution (HBS) and the time of drug addition, respectively. The ATP concentrations measured at 0 min were considered basal values. DMSO, dimethyl sulfoxide.

Figure 1. The time-dependent course of the effects of the transient receptor potential (TRP)M8 agonist menthol (A), TRPA1 agonist cinnamaldehyde (B), TRPV2 agonist cannabidiol (C), and TRPV1 agonist capsaicin (D) on ATP release from the rat nasal mucosa. The ATP concentrations with addition of 10 μM capsaicin were 10.3 ± 2.0 fM and 8.25 ± 1.7 fM (n = 12) after 5-min and 10-min applications, respectively, significantly higher than the basal value of 2.17 ± 0.5 fM (*, p < 0.05 in both cases). In contrast, 10 mM menthol (n = 5), 10 mM cinnamaldehyde (n = 5), and 1 μM cannabidiol (n = 6) showed no significant effects on ATP release. The time points of −5 and 0 min represent the soaking of mucosal segments in 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer solution (HBS) and the time of drug addition, respectively. The ATP concentrations measured at 0 min were considered basal values. DMSO, dimethyl sulfoxide.

The effects of a TRPV antagonist ruthenium red (RR) and pannexin-1 blockers carbenoxolone (CBX)\textsuperscript{13-15} and probenecid\textsuperscript{13,14,16} on capsaicin-induced ATP release from the nasal mucosa are summarized in Fig. 2. In the presence of 100 μM RR, capsaicin-induced ATP release was significantly inhibited, declining from 10.3 ± 2.0 fM to 2.71 ± 0.4 fM after 5 min (Fig. 2A; P < 0.05, n = 7). Similarly, in the presence of 10 μM CBX or 300 μM probenecid, the capsaicin-induced increase of ATP concentration significantly decreased to 3.85 ± 0.9 fM or 5.46 ± 1.0 fM, respectively, in 5 min (Fig. 2B; both P < 0.05, n = 13 each). The percent of 10 μM capsaicin-induced ATP release inhibited by after 5 min was 73.8 ± 4.1%, 62.7 ± 9.2%, and 47.1 ± 9.7%, respectively. A 10-min challenge using RR significantly decreased the ATP concentration from 8.25 ± 1.7 fM to 1.86 ± 0.3 fM (Fig. 2A; P < 0.05, n = 7), while 10-min challenges with CBX and probenecid resulted in concentrations of 4.69 ± 0.8 fM and 5.23 ± 1.2 fM, respectively, which were not significantly different than the pre-challenge value (P > 0.05). These results suggest that the release of ATP from the rat nasal mucosa stimulated by capsaicin is mediated, at least
in part, by the pannexin-1 channel. In each group, there were no statistical differences among the basal values. After confirming the tissue viability by Yo-Pro®-1 iodide, data from 17 segments were excluded. All 17 excluded segments had strong green fluorescence in the cell nuclei within, and the average ATP concentration of these segments was 87.4 ± 27 fM (ranging from 9 to 440 fM), which was significantly higher than generated by capsaicin-induced ATP release (P < 0.05). These data suggest that the ATP concentration increase induced by cell membrane destruction is much higher than that induced by the drugs used in the present study.

To directly assess whether TRPV1 functionally associates with pannexin-1 in nasal columnar epithelial cells, we next examined the effects of pannexin-1 blockers on the step pulse application-induced currents with capsaicin in isolated cell bodies (Fig. 3A and B). We examined the effect of a 5-min treatment with 10 μM CBX (Fig. 3C) or 300 μM probenecid (Fig. 3D) in the presence of 10 μM capsaicin on the amplitude of voltage step-evoked currents in a single nasal columnar epithelial cell. The current-voltage relation plots for each condition are shown in Fig. 3E. The averages of peak current densities measured at +60 mV with the same voltage step protocol were 27.8 ± 1.5 pA/pF with 10 μM capsaicin only (n = 8), 15.9 ± 0.9 pA/pF with 10 μM capsaicin + 10 μM CBX (p < 0.05 vs. capsaicin only, n = 4), and 20.3 ± 1.6 pA/pF with 10 μM capsaicin + 300 μM probenecid (p < 0.05 vs. capsaicin only, n = 4). The average resting potential and membrane capacity of the cells tested were −57.8 ± 2.2 mV (ranging from −70 to −52 mV) and 8.43 ± 0.64 pF (ranging from 6.2 to 11.8 pF), respectively (n = 8). All whole-cell configurations maintained a membrane resistance of over 1 GΩ and an access resistance of less than 20 MΩ throughout the recordings.

The effects of capsaicin on CBF in the rat nasal mucosa in the presence or absence of pannexin-1 blockers are summarized in Fig. 4. Using a microscope with a digital high-speed camera (HAS-U1, DITECT, Tokyo, Japan), we identified the cilia (Fig. 4A, arrow heads) and recorded their movement. We analyzed the CBF in a double-checking fashion; images were captured and the
CBF counted by the original observer, B.H.D., and then re-analyzed and approved by a second observer, T.O. Capsaicin (10 μM) significantly increased the CBF (Fig. 4B, 170 ± 10%, P < 0.05 vs. basal value of 100 ± 6.1%, n = 5). In the presence of 10 μM CBX, capsaicin had no effect on the CBF (Fig. 4C, 101 ± 7.5%, P > 0.05 vs. basal value of 100 ± 2.8%, n = 5). Similarly, in the presence of 300 μM probenecid, capsaicin did not
elicit a significant change in CBF (Fig. 4D, 104 ± 2.9%, P > 0.05 vs. basal value of 100 ± 1.6%, n = 5). These results indicate that CBF regulation mediated by pannexin-1 contributes to the observed capsaicin-induced effect in rat nasal mucosa.

**Discussion**

The present study indicates that the TRPV1 agonist capsaicin significantly potentiated ATP release from rat nasal mucosa, while the TRPM8 agonist menthol, TRPA1 agonist cinnamaldehyde, and TRPV2 agonist cannabidiol did not. CBF was also stimulated by capsaicin. Capsaicin-induced ATP release and CBF stimulation were significantly inhibited by pannexin-1 blockers, indicating the involvement of pannexin-1 in this mechanism. Functional pannexin-1 expression was confirmed electro-physiologically in the rat nasal columnar epithelia at the single-cell level.

Recent reports have demonstrated that the excitation of several kinds of TRP channels involves a relationship between pannexin-1 opening and ATP release into the extracellular space, but it is not yet clear how activation of TRP channels can trigger the opening of pannexin-1 pores.10,11 For example, exposure to cigarette smoking caused dose-related ATP release from human primary bronchial epithelial cells, and the increase in ATP release was attenuated by pannexin-1 blockers or a TRPV1/TRPV4 blocker.10 Another report found that ATP was released from mechanically-stimulated odontoblasts via pannexin-1 in response to TRP channel activation, and subsequently transmitted a signal to trigeminal ganglion neurons.11 Here, we found that capsaicin, an agonist of the spice- and heat-sensitive TRPV1 channel, potentiated the release of ATP, which is likely to be mediated by the pannexin-1 expressed in rat nasal columnar epithelial cells.

In our protocol, ATP release induced by capsaicin decreased over time despite the continuous presence of the agonist. Although we were not able to address this hypothesis, one possibility is that the ATP pool and/or synthesis in the tissue was depleted due to the \( \text{Ca}^{2+} \) influx through the pannexin-1 which depolarizes the mitochondrial membrane potentials during capsaicin application; capsaicin-evoked ATP concentrations did not decrease in the presence of the pannexin-1 blockers which prevent the loss of the mitochondrial membrane potentials supports our hypothesis.

Our results clearly indicate that capsaicin-induced ATP release thorough pannexin-1 strongly regulated ciliary movement. However, treatment with either pannexin-1 blocker, 10 \( \mu \text{M} \) CBX or 300 \( \mu \text{M} \) probenecid, completely diminished the effect of capsaicin on ciliary movement increase, but not its effects in other experiments. The reason for this discrepancy is unknown. One possibility is that the ATP concentration in the mucus blanket on the mucosal surface did not reach a threshold of activation for signaling in the presence of the pannexin-1 blockers; however, it is very difficult to determine this concentration. Although it is unclear how activation of TRPV1 triggers the opening of pannexin-1, the regulatory mechanism involved in the effect of capsaicin could be a key mechanism regulating upper airway function. Modulation of these molecules may present a novel therapeutic strategy in the management of upper respiratory disorders such as temperature change- and spicy food-induced nonallergic vasomotor rhinitis, whose pathophysiological mechanisms have not yet been elucidated.

**Materials and methods**

Thirty-eight young adult male Wistar rats (Kyudo) aged 4–6 weeks were used in this study. The animals were housed in standard plastic cages at 23–25°C in a 12-hour light/dark cycle. After cervical dislocation, the animals were decapitated. The dorsum nasi was surgically opened at the midline, and the mucosa at the common nasal meatus was collected. The collected filmy mucosa was trimmed using a circular punch (inner diameter = 1.8 mm) and blade, and then pre-incubated in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (HBS) at room temperature (22–24°C) for 1 h before use. HBS contained 140 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 2 mM CaCl2, 1.2 mM MgCl2, 10 mM glucose, and 10 mM HEPES, was adjusted to pH 7.4 with Tris base, and was continuously oxygenated with 100% O2.

To measure ATP, the excised mucosal segments were placed individually in wells of 12-well culture plates containing 4 ml culture medium and maintained in a humidified incubator at 37°C with 5% CO₂ for 24 hours. The culture medium used was Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Gibco/BRL), 0.1 mM 2-mercaptoethanol (Wako), 0.1 mM nonessential amino
acids (Gibco/BRL), 1 mM sodium pyruvate (BioWhit-taker Molecular Applications), and 1% penicillin-streptomycin (Sigma). After washing, the mucosal segments were placed with 4 ml HBS into the wells of another 12-well culture plate at room temperature. A 100-μl sample of buffer was collected from each well by an AQUASNApTM ATP water-testing device (Higyiena), and the ATP concentration measured by a luciferin-luciferase assay every 5 min for 15 min, including a basal 5-min incubation, using the System-SURE PlusTM luminometer (Higyiena) as described previously.18 To assess the contribution of TRP channels to ATP release, the mucosal segments were incubated in the presence of TRP agonists at room temperature: 10 mM menthol for TRPM8, 10 mM cannaldehyde for TRPA1, 10 μM capsaicin for TRPV1, or 1 μM cannabidiol for TRPV2 (all from Sigma). The mucosal segments were also incubated in the presence of 100 μM RR, 10 μM CBX, or 300 μM probenecid (all from Sigma) in combination with 10 μM capsaicin.

After ATP measurement, all segments were soaked in the dead-cell indicator Yo-Pro®-1 iodide (Thermo Fisher Scientific) (×5000) for 30 s, and then observed under a phase-contrast microscope with a green fluorescent filter to determine the viability of the tissues. We previously reported that distinct fluorescence for pannexin-1 was observed in the epithelial layer by fluorescence immunohistochemistry.9 Based upon these results, the slices that responded to Yo-Pro®-1 in both the epithelial layer and the submucosal layers were considered non-viable tissues to exclude Yo-Pro uptake through functional pannexin-1 in a fully open state.

CBF was measured on segments of nasal mucosal epithelium using a digital high-speed camera (HAS-U1 [DITECT, Tokyo, Japan]) microscopy system, within 2 h of sample collection. The high-speed video images were analyzed in a double-checking fashion as described above in the Results.

Patch clamp experiments were performed on rat nasal columnar epithelial cells that were dissociated by an enzymatic digestion method19 with minor modification. Briefly, the nasal mucosa segments were incubated in 4 ml HBS containing DNase I (0.5 mg ml⁻¹; Sigma) and papain (20 U ml⁻¹; Worthington Biochemical Corp.) in a 50-ml Eppendorf tube with 100% oxygen for 10 min at 30°C. The segments were then transferred to normal HBS and washed for at least 1 h before mechanical dissociation by repeatedly drawing up and dispensing samples with fire-polished glass pipettes. The cell suspension was plated onto coverslips placed individually in the wells of multi-well culture plates with 4 ml culture medium as described above and maintained in a humidified incubator at 37°C with 5% CO₂ for 24 hours. The coverslip was then placed in a glass-bottomed chamber and continuously perfused with HBS at a rate of 1.5 ml min⁻¹ using an 8-head peristaltic pump (MP-8; Gilson nucleus). The solution level was kept constant by a low-pressure aspiration system. Isolated columnar epithelial cells were identified by their morphology (Fig. 4A). The electrodes used in this study were triple-pulled from a glass capillary with a puller (P-87; Sutter Instrument Co.). The pipettes had a final resistance of 5–8 MΩ when filled. The pipette solution used in the recording electrodes contained 140 mM potassium gluconate, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM HEPES, and 2 mM Mg-ATP, adjusted to pH 7.3 with Tris base. An axopatch 200B amplifier (Axon Instruments) was used to record ionic currents. Membrane voltages and currents were controlled and recorded with a computer running pCLAMP10.3 software (Molecular Devices). Recording commenced at least 3 min after membrane rupture when the currents reached a steady-state. Recordings included in data analysis were collected during periods of stable series resistance. All electrophysiological experiments were performed at room temperature (22–24°C). Currents elicited by each voltage command in the tight-seal, whole-cell configuration were obtained by applying a series of 500-ms voltage command steps every 1 s, from a holding potential of −60 mV to different potentials (−30 to +60 mV) at 15-mV increments. Drugs were applied in the bath perfusion.

Results are expressed as means ± SEMs. Statistical comparisons were performed with a 2-tailed Student's t-test or Welch's t-test.

All experiments were performed in accordance with the guidelines of the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health (UOEH). All field methods were performed in accordance with procedures that were approved by the Laboratory Animal Research Center, UOEH, JAPAN, 2016 Sixth Edition.
(assignment numbers: LA16–12 for T.O. and LA16–50 for B.H.D.).

**Abbreviations**

- **CBF**: ciliary beat frequency
- **CBX**: carbenoxolone
- **EGTA**: ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
- **HBS**: HEPES buffer solution
- **HEPES**: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- **RR**: ruthenium red
- **TRP**: transient receptor potential

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author contributions**

T.O. and H.S. designed the research; T.O. and B.H.D. performed the research, coordinated the study, participated in data collection, and wrote the paper together with H.K., S.T., Y.U., and H.S. All authors discussed the results, commented on the manuscript, and approved the version to be published.

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