Biochemical and Biophysical Research Communications 463 (2015) 1006–1011

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Nucleotide modulates odor response through activation of purinergic receptor in olfactory sensory neuron

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ARTICLE INFO

Article history:
Received 7 May 2015
Accepted 7 June 2015
Available online 10 June 2015

Keywords:
Olfactory sensory neuron
Purinergic receptor
Nucleotide
Calcium imaging

ABSTRACT

Extracellular nucleotides are important neurotransmitters, neuromodulators and paracrine factors in the neural sensory system [16]. Most of purines and pyrimidines act on the associated purinergic cell-surface receptors to mediate sensory transduction and modulation. Previously, we reported a subgroup of heptaldehyde (H)/2-hepatanone (Ho)-responsive olfactory sensory neurons (H/Ho-OSNs) in the ventral endoturbinates [31]. Through the calcium image recording, we characterized that ATP elicited $[Ca^{2+}]_i$ increase in the presence of extracellular calcium, while depletion of intracellular calcium stores blocked UTP-evoked $[Ca^{2+}]_i$ increase. Pharmacological studies indicated that $P2X_3$ was expressed in the H/Ho-OSNs, modulating both heptaldehyde (H) and 2-hepatanone (Ho)-induced responses. These data indicated that activation of purinergic receptor negatively modulated odor response, providing the evidence to support the possible protective effect of purinergic receptor in OSNs.

1. Introduction

Purinergic nucleotides are important extracellular signaling molecules involved in peripheral visual [25], olfactory [27,8], auditory [20] and gustatory sensory system [5]. They act on purinergic receptors to play vital roles in development, neurotransmission, neuromodulation and neuropathology [2,4,16]. Two types of purinoceptors are identified as P1 and P2 [1]. P1 receptors are subdivided into four types, A_1, A_{2A}, A_{2B}, A_3 [1]. P2 receptors are composed of P2X and P2Y subtypes. P2X receptor family comprises seven subtypes ($P2X_1$–$P2X_7$), all of which are ligand-gated ion channel receptors [22]. P2X receptors form intrinsic Ca^{2+}-permeable nonselective cation channel and allow Ca^{2+} influx from extracellular space [22,19]. Until now, eight P2Y receptor subtypes are recognized. Most of P2Y receptors are G-protein coupled receptors, through which intracellular secondary messenger (cAMP or IP_3) is produced, and then calcium is mobilized and released from intracellular stores [1,7].

In vertebrates, the olfactory epithelium is the primary site to sense the odor. The olfactory epithelium is composed of 6–10 million of olfactory sensory neurons (OSNs). Each OSN possesses an olfactory receptor (OR), which is a G-protein coupled seven -transmembrane receptor [21,28]. OSNs respond to odors with OR-mediated generation of intracellular secondary messengers, and then open cyclic nucleotide-gated (CNG) channels, allowing influx of calcium [6]. Clearly, Ca^{2+} is vital as a third messenger in the ofactory transduction [26].

Recent studies indicated that multiple purinergic receptor subtypes are expressed in the mouse olfactory epithelium, such as $P2X_1$, $P2X_4$, $P2Y_2$ in the OSNs [13], $P2Y_4$ in the sustentacular cells [29], and $P2X_1$, $P2Y_2$ in the basal cells [13]. It has also been suggested that extracellular ATP induced a suppression of activity of OSNs, and activation of P2X and P2Y modulated odor sensitivity [13]. Besides, purinergic receptor antagonists decreased the proliferation rate of basal cells in the tadpole olfactory epithelium [12], and it blocked induction of heat shock protein 25 in mouse olfactory epithelium [15]. Activation of purinergic receptors induced proliferation, neuronal differentiation, and neuropeptide Y release in mouse olfactory epithelium [17,18]. All these researches provided a clue that activation of purinergic receptors may initiate protective signaling pathways when OSNs are overexposed to odors or toxicants.

Nucleotides-induced calcium responses have been characterized in sustentacular cells and basal cells of tadpole olfactory epithelium [11]. Here, we identified the purinergic receptor subtypes and characterized the nucleotides-evoked calcium signaling in heptaldehyde (H)/2-hepatanone (Ho)-responsive OSNs (H/Ho-

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http://dx.doi.org/10.1016/j.bbrc.2015.06.050
0006-291X/

OSNs). We also determined the role of nucleotides in regulating the odor responses in this subgroup of OSNs. Furthermore, we elucidated the possible interaction between odor response and activation of purinergic receptor in OSNs. This may facilitate us better understanding the mechanism involved in modulation of odor sensitivity via neurotransmitters, such as ATP.

2. Materials and methods

2.1. Chemicals and solutions

All odorants used here were purchased from Sigma Aldrich (St Louis, MO). They were freshly made to 100 μM each by directly diluted in Ringer's saline containing 145 mM NaCl, 5 mM KCl, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM Na pyruvate, and 5 mM D-glucose. Ca2+-free solution was made by omitting $CaCl_2$ in saline plus 3 mM ethylene glycol tetraacetic acid (EGTA). All solutions were adjusted to pH 7.4 and oxygen-saturated before use.

Uridine 5′-diphosphate (UDP), adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate (ATP) adenosine and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) were purchased from Sigma. Uridine 5′-triphosphate (UTP) was purchased from Fermentas (Glen Burnie, MA). α,β-methylene ATP (α,β-meATP), Uridine-5′-(γ-thio)-triphosphate (UTPγS) trisodium salt, 2-thiouridine 5′-triphosphate (2-ThioUTP) tetrasodium salt, suramin, RO-3, NF449, ARL67156 trisodium salt, and BAPTA AM were purchased from TOCTIS bioscience (Ellisville, MI). All nucleotides were dissolved in concentrated solutions to 100 mM. The stock concentration of ARL67156 and BAPTA AM were 50 mM. PPADS and suramin were 20 mM. RO-3, and NF449 were 10 mM. All chemicals were diluted with 1000 times in Ringer's solution just before use.

2.2. Preparation of intact mouse turbinates

All procedures of animal handling were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the Illinois Institute of Technology. Adult C57BL/6 mice at 2–3 months of age were used in the experiments. The decapitated mouse head was opened along the mid line, and the endoturbinates were exposed by removing the septum. The olfactory bulb and bones around ectoturbinates were removed and the turbinates were loaded with calcium sensitive dye Fura-2 AM (Invitrogen, Carlsbad, CA) similar to described before [24]. In brief, the turbinates were incubated in 10 μM fura-2 AM and 0.02% nonionic dispersing agent Pluronic F-127 at 37 °C for 1 h. The turbinates were mounted to a recording chamber with endoturbinates facing up and were continuously perfused with oxygenated saline throughout the experiments.

2.3. Calcium imaging and data evaluation

Ratiometric calcium imaging recording was performed at excitation of 340 nm (F340) and 380 nm (F380) in an Olympus upright microscope (BX51WI) equipped with a 20x, 0.9 numerical aperture water immersion objective, a filter wheel (Sutter Instruments, Novato, CA), a 175w xenon lamp and a cooled CCD camera (SensiCam qe; Cooke Corporation, Romulus, MI). Images were collected every 4 s using Imaging Workbench 5.2 (Indec Biosystems, Santa Clara, CA). Data were binned every four frames after recording using the Excel program and presented in the ratio of F340/F380 (Fr). The olfactory response magnitude of individual OSN ΔF/F was calculated as (Fr − F)/F, where Fr was the response to a stimulus at any time point and F was baseline activity, obtained by averaging 10 frames before stimulation. Data were presented as mean ± SEM. Paired Student's t-tests were used to determine significant differences. A p value less than 0.05 is considered as different and highly different if p was less than 0.01.

3. Results

3.1. Responsiveness to purinergic agonists in H/Ho-OSNs

Previous studies indicated that several purinergic receptor subtypes were expressed in olfactory sensory neurons [13,8]. To determine which purinergic receptor subtype(s) were expressed in H/Ho-OSNs located in the ventral region of olfactory epithelium, a variety of purinergic agonists activating different purinergic receptor subtypes were applied to this subgroup of neurons. We found that H/Ho-OSNs respond to ATP (a nonselective purinergic agonist), UTP (a nonselective P2Y receptor agonist), and α,β-MeATP (a $P2X_1/P2X_3$ receptor agonist) (Fig. 1A). ATP responsive potency was always greater than that of UTP or α,β-MeATP (Fig. 1A). These data showed that both P2X and P2Y receptors were present in the H/Ho-OSNs. However, adenosine, which is known to activate P1 receptor [1], did not elicit a Ca^{2+} transient (Fig. 1A). Also, ADP or UDP potency was weaker than that of ATP or UTP (Fig. 1A). Furthermore, Both UTPγS (a selective $P2Y_2/P2Y_4$ receptor agonist) and 2-ThioUTP (a selective $P2Y_2$ receptor agonist) can lead to intracellular calcium increase in the H/Ho-OSNs (Fig. 1A). 2-ThioUTP response is generally more potent than that of UTPγS, suggesting the expression of $P2Y_2$. However, this did not exclude the possibility of $P2Y_4$ expression in the H/Ho-OSNs. Collectively, our results indicated that $P2X_1/P2X_3$ and $P2Y_2/P2Y_4$ receptors were expressed in the H/Ho-OSNs.

3.2. ATP and UTP evoked intracellular calcium increases via different Ca^{2+} sources

Transient intracellular calcium increase occurs through calcium influx from extracellular space, or intracellular calcium store release [23]. We investigated the calcium sources to the purinergic stimulus in the H/Ho-OSNs through application of Ringer's salt without calcium and with cytosolic calcium chelator BAPTA AM. ATP-evoked calcium response was significantly inhibited without extracellular calcium in H/Ho-OSNs. Repeated applications of ATP in Ca^{2+}-free Ringer's solution resulted in gradual rundown of the Ca^{2+} responses (First: 72.7 ± 2.5% of control, $p < 0.01$; Second: 52.6 ± 2.5% of controls, $p < 0.0001$; Third: 42.2 ± 2.4% of controls, $p < 0.0001$, $n = 10$, Fig. 1B). Returning to standard Ringer's solution did not fully recover the ATP response (71.9 ± 3.9% of control, Fig. 1B). UTP-evoked calcium response was not blocked in the absence of extracellular calcium (94.9 ± 4.2% of control, $p > 0.1$, $n = 10$, Fig. 1C). By contrast, superfusion of BAPTA AM significantly reduced UTP-induced calcium transient by 55.3 ± 4.7% ($p < 0.0001$, Fig. 1C). To sum up, our data provided evidences to support that ATP-evoked calcium response required extracellular calcium, whereas UTP-induced calcium transient was predominately mediated by calcium release from intracellular stores.

3.3. ATP-induced Ca^{2+} transient was specific and correlated to purinergic receptors

To determine the specificity of ATP-evoked intracellular calcium increase, the dose dependency of ATP-induced $[Ca^{2+}]_i$ increases in the H/Ho-OSNs was shown in Fig. 2A. EC_{50} value of ATP-induced calcium transients was 114.3 μM. Then, we examined the effect of two non-selective P2 receptor antagonists, Suramin and PPADS, on ATP-evoked calcium response. Both Suramin and PPADS superfusion effectively inhibited ATP-induced $[Ca^{2+}]_i$ increase in H/Ho-

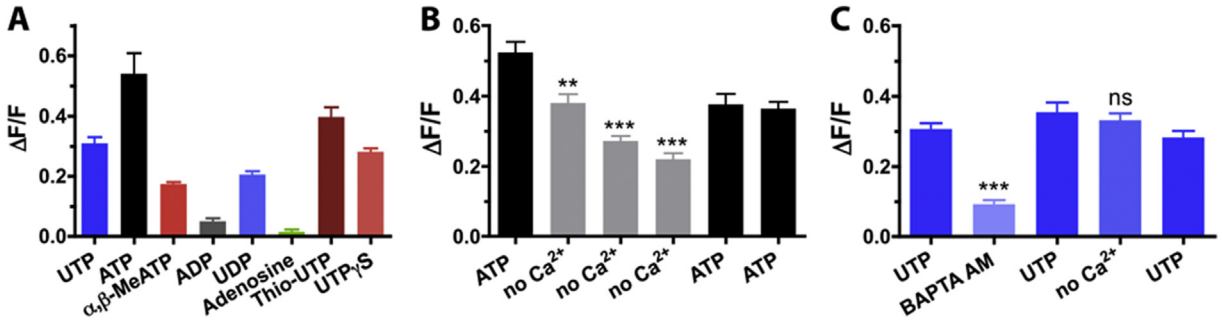

Fig. 1. Both P2X and P2Y receptors are expressed in the H/Ho-OSNs. (A) Response profile to different nucleotides. (B) Extracellular calcium was required in ATP-evoked calcium transient. (C) UTP-induced calcium response did not require extracellular calcium. In contrast, calcium release from intracellular stores was necessary.

OSNs (Fig. 2B). ATP-induced calcium response was reduced to 24.3 ± 1.2% of control ($n = 14$, $p < 0.0001$) in the presence of PPADS, and to 22.7 ± 1.8% of control ($n = 14$, $p < 0.0001$) with application of suramin. We also tested the selective ecto-ATPase inhibitor ARL67156 on ATP-evoked calcium transient in H/Ho-OSNs. Application of ARL67156 increased calcium response by 54.3 ± 10.4% ($n = 13$, $p < 0.01$, Fig. 2C and 2D). This indicated that inhibition of ATP degradation significantly enhanced the effect of ATP on triggering intracellular calcium transient. These data suggested that ATP-induced calcium transient in the H/Ho-OSNs was resulted from the activation of P2 receptors.

3.4. Purinergic agonists modulate odor responses in the H/Ho-OSNs

Previous studies showed that nucleotides played a role in modulation of odor response, which may imply a protective mechanism for OSNs when they are overexposed to odors or chemicals [13]. Here, our data indicated that purinergic agonists were able to regulate odor responses in the H/Ho-OSNs. We sequentially superfused 2-hepatanone (Ho), purinergic agonists (ATP or α,β-MeATP) and dose of combination, and found that the co-application of ATP and Ho decreased the Ca^{2+} transients by 45.2 ± 2.7% compared with the predicted additive amplitudes ($n = 6$, $p < 0.01$, Fig. 3A and 3B). Similarly, co-application of the P2X agonist α,β-MeATP and Ho suppressed the Ca^{2+} transient amplitude by 46.5 ± 1.5% of predicted peak ($n = 6$, $p < 0.01$, Fig. 3C and 3D). Moreover, the suppression caused by co-application was neither the result of unhealthy condition of cells because difference between responses to nucleotides and to Ho before and after co-application was within 15%, nor the result of dye saturation since peak amplitude of 100 μM Ho, 100 μM ATP and the dose of combination fell well into the linear range of Ho dose–response

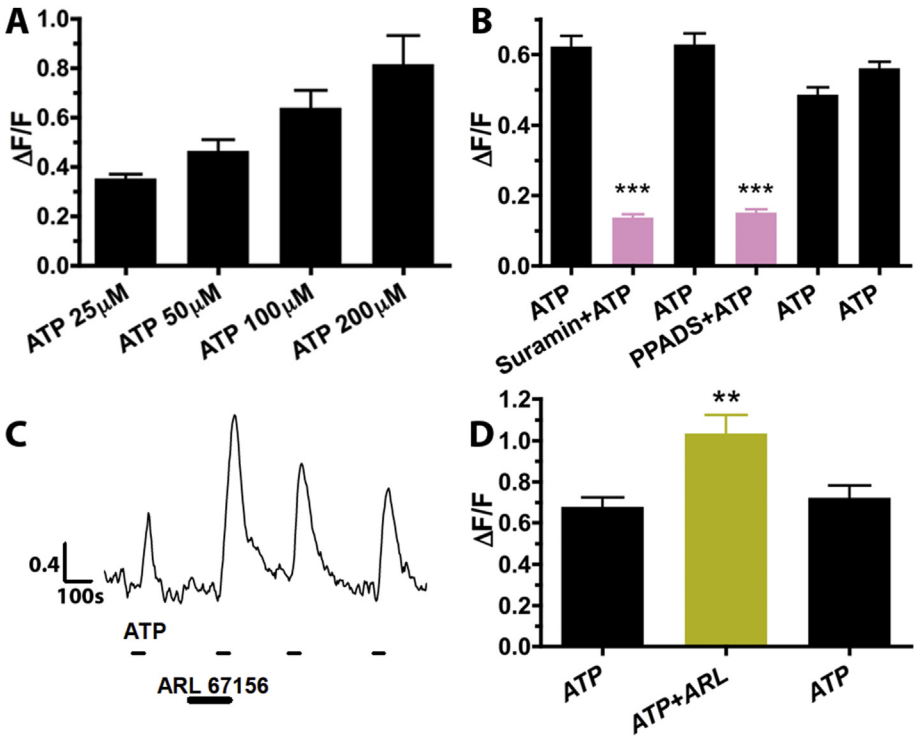

Fig. 2. ATP acted on purinergic receptors to evoke intracellular calcium response in the H/Ho-OSNs. (A) Dose-dependent response to ATP. (B) Non-selective purinergic antagonists, PPADS and suramin blocked ATP-induced intracellular calcium response. (C) Application of ARL67156 increased ATP-evoked calcium transient. The quantitative data and statistical analysis are shown in (D). $^{**}p < 0.01$, $^{***}p < 0.001$, student's t-tests.

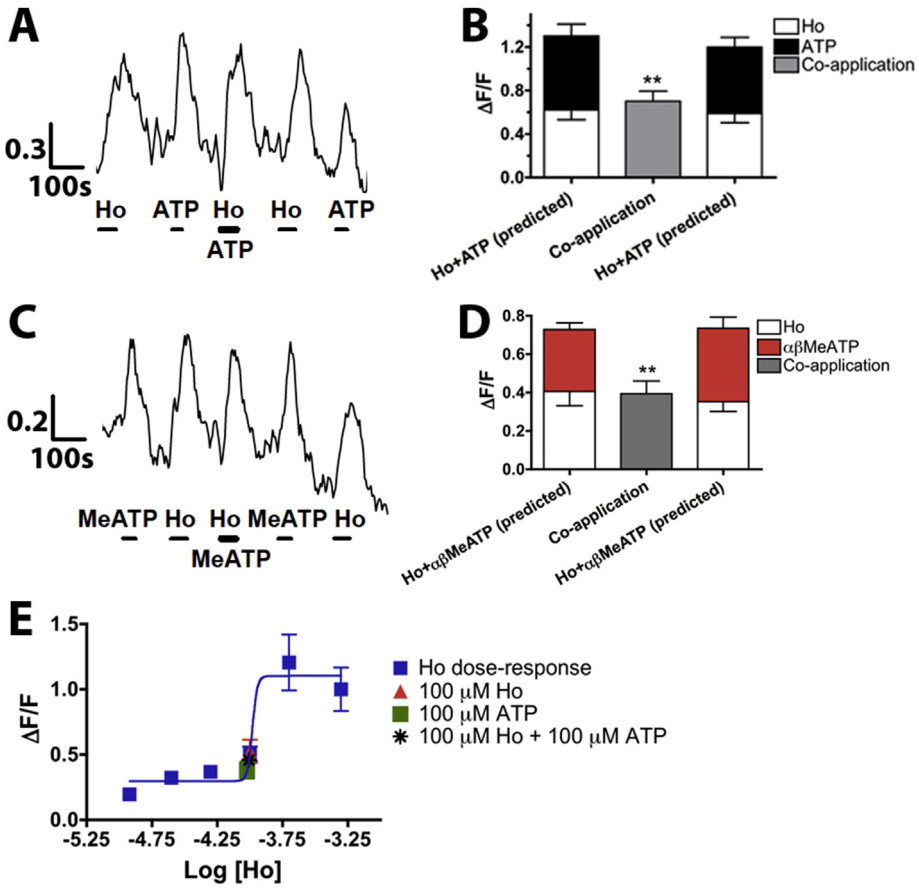

Fig. 3. Purinergic agonists modulated odor response in the H/Ho-OSNs. (A) ATP modulated 2-hepatanone-evoked response. The quantitative data and statistical analysis were shown in (B). (C) α,β-MeATP modulated 2-hepatanone-evoked response. The quantitative data and statistical analysis were shown in (D). (E) Dose-response relationships for hepatanone-induced calcium transients indicated that odor, nucleotide, and co-application-evoked calcium transients were within the linear range. $^{**}p < 0.01$, student's t-tests.

relationship (Fig. 3E). Thus, we came to the conclusion that activation of purinergic receptor was able to modulate odor response in the H/Ho-OSNs.

3.5. Activation of purinergic receptor specifically and negatively modulate odor response

To elucidate the underlying mechanism of differential modulation of odor sensitivity in single OSNs, we determined specific interaction between odor response modulation and purinergic receptor activation. We tested the selective purinergic antagonists in the H/Ho-OSNs. The results indicated that Ro-3, a selective $P2X_3$ antagonist increased H-evoked calcium transient by 99.3 ± 7.8% ($n = 6$, $p < 0.01$, Fig. 4A). By contrast, application of NF449 (a selective $P2X_1$ antagonist) did not increase the H-induced calcium transient (110 ± 5.4% of control, $n = 6$, $p > 0.1$, Fig. 4A). Similarly, NF449 did not block the Ho-induced calcium transient (115.0 ± 9.5% of control, $n = 6$, $p > 0.1$, Fig. 4B), while Ro-3 increased Ho-evoked calcium transient by 26.9 ± 3.1% ($n = 6$, $p < 0.05$, Fig. 4B). Then, we asked if there is chemical interaction between nucleotide and odor. We did cross-adaptation experiments to confirm that heptaldehyde (H) or 2-hepatanone (Ho) did not compete for the ATP-binding site by measuring ATP-evoked response during perfusion of heptaldehyde (H) or 2-hepatanone (Ho). As shown in Fig. 4C and 4D, $[Ca^{2+}]i$ transient evoked by ATP did not change significantly during adaptation of odors, indicating there was minimal chemical interaction between ATP and odor at the receptor level ($n = 9$, $p > 0.1$). Thus, heptaldehyde (H) and 2-hepatanone (Ho)-evoked responses in single H/Ho-OSNs were negatively modulated by $P2X_3$ receptor.

4. Discussion

4.1. Expression of purinergic receptors in OSNs

Our findings indicated that both P2X and P2Y receptors, but not P1 receptor were present in the H/Ho-OSNs. We also determined the subtypes of purinergic receptors in these OSNs. Application of α,β-MeATP elicited intracellular calcium response, suggesting preferential expression of $P2X_1$ and $P2X_3$ [1,22]. Amplitiude of α,β-MeATP-evoked calcium transient was a bit smaller than that of ATP, indicating absence of $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_7$ in these OSNs since the reported agonist ranking order for these receptors was ATP >>α,β-MeATP [1,22]. Besides, application of phenolphthalein (a selective $P2X_4$ antagonist) and chelerythrine (a selective $P2X_7$ antagonist) did not reduce ATP-evoked calcium transient (data not shown), which was a further indication against the involvement of $P2X_4$ and $P2X_7$ in calcium response. Moreover, the calcium response elicited by ATP was partially blocked by superfusion of Ro-3 (a selective $P2X_3$ antagonist), demonstrating the participation of this subtype in calcium signaling in the H/Ho-OSNs (data not shown).

We also found UTP can evoke $[Ca^{2+}]_i$ increase in the H/Ho-OSNs, showing the involvement of P2Y receptors in calcium response. Our determined agonist ranking was ATP > UTP > UDP > ADP (Fig. 1A) so that $P2Y_1$ (ADP > ATP), $P2Y_6$ (UDP > UTP >> ATP), $P2Y_{12}$

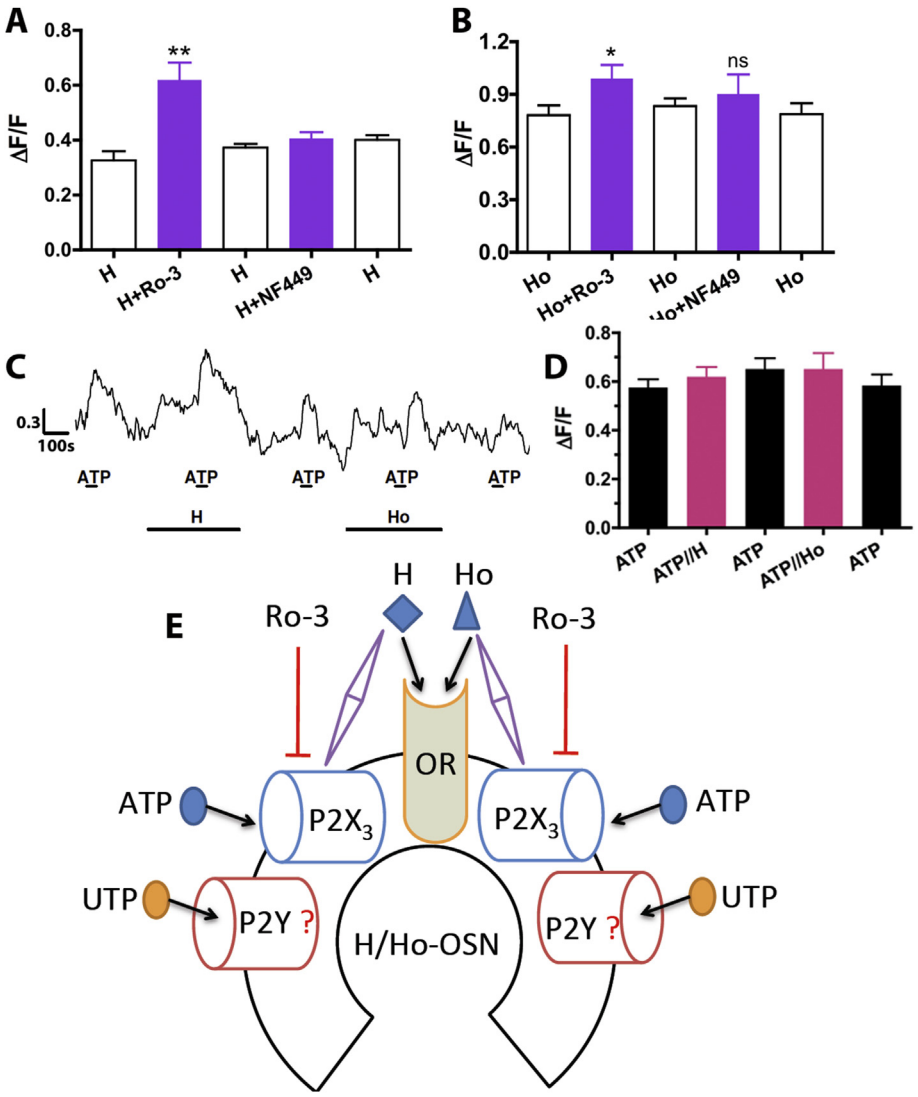

Fig. 4. Specific interaction between odor response modulation and purinergic receptor activation. (A) Heptaldehyde-induced calcium transient was increased in the presence of $P2X_3$ receptor antagonist, but not $P2X_1$ receptor antagonist. (B) 2-hepatanone-induced calcium transient was increased in the presence of $P2X_3$ receptor antagonist, but not $P2X_1$ receptor antagonist. (C) A sample trace of calcium imaging showed ATP-evoked $[Ca^{2+}]i$ responses during adaptation of heptaldehyde (H) or 2-hepatanone (Ho). The quantitative data and statistical analysis were shown in (D). (E) A diagram predicting modulation of the olfactory receptor (OR) through $P2X_3$ receptor in the H/Ho-OSNs. $^{*}p < 0.05$, $^{**}p < 0.01$, student's t-tests.

(ADP >> ATP), or $P2Y_{13}$ (ADP >> ATP) was not expressed in these OSNs [1,22]. Besides, UTP-induced calcium response was not blocked in the presence of MRS2365 (a selective $P2Y_1$ antagonist), further excluding the expression of $P2Y_1$ in these OSNs (data not shown). Moreover, both UTPγS (selective $P2Y_2$/$P2Y_4$ agonist) and 2-ThioUTP (selective $P2Y_2$ agonist) elicited calcium transients in these OSNs, and ranking order was 2-ThioUTP > UTPγS ≥ UTP (Fig. 1A), providing evidences for expression of $P2Y_2$ receptor. Thus, we came to the conclusion that $P2X_3$ and $P2Y_2$ (or $P2Y_4$) were expressed in the H/Ho-OSNs.

4.2. Calcium source from intracellular store or extracellular space

Several nucleotides activate P2X and P2Y receptors, and stimulate $[Ca^{2+}]_i$ increase [30,22]. P2X receptors form nonselective cation channels, and Ca^{2+} influx through the pore of these channels accounts for the increase in $[Ca^{2+}]_i$ [22,19]. P2Y couples to G-protein to induce activation of phospholipase C and generation of IP_3, then mobilizing the release of calcium from internal stores [30]. In this study, we characterized ATP and UTP-evoked calcium increase in the H/Ho-OSNs. The amplitude of ATP-induced calcium transient decreased in absence of extracellular calcium (Fig. 1B), indicative of activation of P2X receptor via ATP stimulation. This was not consistent with the results obtained in sustentacular supporting cells (SCs) of the tadpole olfactory epithelium [11]. The difference may result from the sole expression of P2Y receptor in SCs. In contrast, UTP-induced calcium transient was not affected in the absence of extracellular calcium (Fig. 1C), suggesting activation of P2Y receptor by UTP. BAPTA AM blocked UTP-induced calcium transient, suggestive of calcium release from intracellular store. The similar results were observed in SCs of the mouse olfactory epithelium [14].

4.3. Modulation of different odor sensitivity via various nucleotides in single OSNs

Hegg and his colleagues found that ATP can suppress odor response in OSNs [13]. The possible mechanism was the alteration

of calcium homeostasis with the activation of purinergic receptors in OSNs. It was also reported that olfactory receptor M71 interacts with other G protein-coupled receptors such as β2-adrenergic, $P2Y_1$, $P2Y_2$, and adenosine A_{2A} receptors [3,9,10]. Based on these studies, we investigated modulation of differential odor sensitivity in single OSNs. The previous study determined the agonist ranking order for $P2X_1$ was ATP = α,β-MeATP = 2-MesATP, and for $P2X_3$ is 2-MesATP > ATP> α,β-MeATP [1,22]. This order indicated that both ATP and α,β-MeATP were potent agonist to $P2X_1$, but weak agonist to $P2X_3$. Our study indicated that ATP and α,β-MeATP moderately regulated H and Ho sensitivity in similar pattern (Fig. 3). This was possibly due to interaction between H as well as Ho response with $P2X_3$ receptor, which was certified through antagonist experiment (Fig. 4). UTP, UTPγS and 2-ThioUTP activated the $[Ca^{2+}]$i increase in H/Ho-OSNs. According to the reported P2Y agonist ranking order, the subtype was $P2Y_2$ or $P2Y_4$ [1,30]. However, since we did not find highly selective antagonist to $P2Y_2$ and $P2Y_4$, we did not determine the specific P2Y receptor subtype.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.050.

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