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The role of connexin 43 in mediating odor response

Yiqun Yu*, Chunbo Zhang

Department of Biological and Chemical Sciences, Illinois Institute of Technology, Chicago, IL 60616, USA

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ABSTRACT

Connexin proteins are the hemichannels that form gap junctions to regulate the intercellular communication. Connexin 43 (Cx43) is the most common gap junction protein that expresses in many cell types, including the olfactory sensory neurons. Phosphorylation is a crucial step to regulate the function of Cx43. Gap junction was found to modulate the odor response, but the specific role is still elusive. Here, we report that gap junctions play a role in odor-evoked calcium response in both heterologous cell system and primary olfactory sensory neurons. This regulation is mediated through gap junction protein Cx43. Overexpression of full length Cx43 can counteract the inhibitory effect of gap junction or connexin blockers on odor-evoked $[Ca^{2+}]i$ increase in hana3A cells. Carboxy-terminal of Cx43 (Cx43CT) has the similar function as the full length of Cx43. Furthermore, we found that expression level of phosphorylation of Cx43 at S368 is dynamic with the stimulation of odor in hana3A cells. Expression level of phosphorylated Cx43 at S368 was decreased when gap junction or connexin inhibitors were applied. Phosphorylation of Cx43 during odor or inhibitor stimulation may be mediated by ERK and JNK signaling pathway. Altogether our data suggest that expression of Cx43 can regulate the odor response. This study provides a clue to indicate the possible protective mechanism of gap junction in odor response.

Introduction

Gap junctions are transmembrane channels located on the plasma membranes of adjacent cells that facilitate intercellular communication via exchange of small molecules, such as Ca^{2+} and ATP. Gap junctions play vital roles in many biological functions, such as neural transmission, immune reaction, or reproductive function. Gap junction channels are composed of two hemichannels called as connexons, which connect across the intercellular space. A hexameric complex of protein named connexin forms each connexon. The connexin gene family is comprised of 20 members in the mouse and 21 members in the human genome, and named according to their molecular weights (Söhl and Willecke, 2004). Connexin 43 (Cx43) is the predominant protein forming gap junctions. Cx43 signaling is central to many physiological functions, such as cardioprotection (Boengler et al., 2006), diabetic wound healing (Bajpai et al., 2009), spermatogenesis (Sridharan et al., 2007), cell survival and death (Rodríguez-Sinovas et al., 2007). Recently, connexins were found to be present in olfactory system. Mature olfactory sensory neurons (OSNs) express Cx43 (Zhang et al., 2000), Cx45 (Zhang and Restrepo, 2002), Cx36 (Zhang and Restrepo, 2003), and Cx57 (Zhang, 2011). These studies implicate a potential role of gap junction proteins in olfactory sensory system. However, the function of gap junction proteins in odor coding and information processing in olfactory system is sill elusive. It was reported that olfactory response is not affected by the coupling of OSNs by gap junctions (Delay and Dionne, 2003). In contrast, some clues counteract this concept. A possible role of connexin30.3 in olfaction was characterized in connexin30.3-deficient mice (Zheng-Fischhöfer et al., 2007). Besides, gap junction proteins are able to modulate odor response in OSNs (Zhang, 2010). These studies provide evidence to support that gap junctions are likely to play a role in regulation of odor sensitivity in receptor neurons. One of the vital processes to regulate the cell-cell interaction is the phosphorylation of gap junction proteins. Phosphorylation of Cx43 depends on protein kinases, such as tyrosine protein kinase, MAP kinase, and protein kinase C (Lampe and Lau, 2004). In this study, a heterologous cell system (hana3A cell line derived from HEK293 cell line) is applied to achieve the surface expression of olfactory receptor Olfr62 (Zhuang and Matsunami, 2008). We found that gap junction or connexin inhibitors can block the odor response in heterologous cell system and primary OSNs. By contrast, overexpression of Cx43 in hana3A cells counteracts this inhibitory effect. Besides, Carboxy (C)-terminal of Cx43 (Cx43CT) also functions in preventing

* Corresponding author at: Department of Biological and Chemical Sciences, Illinois Institute of Technology, 3101 S. Dearborn Street, Rm 182 LS, Chicago, IL 60616, USA. Tel.: +1 312 567 8925; fax: +1 312 567 3494.
E-mail address: yyu17@iit.edu (Y. Yu).

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depletion of odor response from application of gap junction or connexin inhibitors. Furthermore, kinetic phosphorylation of Cx43 at S368 is observed through odorant stimulation. Application of connexin inhibitors prevents the phosphorylation of Cx43 at S368 induced by odor stimulation, which is mediated through ERK and JNK signaling.

Materials and method

Chemicals

All odors used here including 2-hepatanone, heptaldehyde, eugenol, and 2-coumaranone were purchased from Sigma (St Louis, MO). They were dissolved in DMSO to make stock solution, then freshly made by dilution in Ringer's saline, which is made of 145 mM NaCl, 5 mM KCl, 20 mM Hepes, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM Na pyruvate, and 5 mM D-glucose. To make the Ca^{2+}-free solution, $CaCl_2$ was replaced with 3 mM ethylene glycol tetraacetic acid (EGTA) in saline. All solutions were adjusted to pH 7.4 and oxygen-saturated before use. Flufenamic acid (FFA), 18-beta-Glycyrrhetinic acid (BGA) and glycyrrhizic acid (GA) were purchased from Fluka. Gadolinium(III) chloride ($GdCl_3$), MDL12330A were purchased from Sigma.

Cell culture and transfection

Hana3A cell line is a gift from Dr. Matsunami (Duke University). Cells were grown in minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), added with 100 μg/ml penicillin-streptomycin, 1.25 μg/ml amphotericin, and 1 μg/ml puromycin. Olfr62 receptor, RTP1S, CRE-Luciferase, and SV40-Renilla constructs are gifts from Dr. Matsunami (Duke University). EGFP-N1 construct is a gift from Dr. Xiang (Illinois Institute of Technology). Full-length connexin 43 constructs were cloned from cDNA of C57BL/6 mice. Sequence encoding for the C-terminal amino acids 242–382 of connexin 43 was cloned into a pcDNA3.1 vector to generate Cx43CT. All constructs were transfected into hana3A cells by using lipofectamine 2000 (Invitrogen). Cells were analyzed 24 h after transfection.

Single cell calcium imaging

Hana3A cells were seeded on cover glasses 24 h before transfection. Transfected cells were incubated in 5 μM fura-2 AM containing 0.02% nonionic dispersing agent Pluronic F-127 at 37 °C for approximately 40 min. The cover glasses were then mounted to a recording chamber (a modified RC-22C, Warner Instrument, Hamden, CT), and were continuously perfused with oxygenated saline throughout the experiments.

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 water immersion objective (0.9 numerical aperture), a filter wheel (Sutter Instruments, Novato, CA), a 175 W 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 presented as the F340/F380 (Fr). The response amplitude ΔF, was calculated as $\Delta F = F - F0$, where F was the peak of Fr responding to a stimulus and F0 was the Fr before stimulation.

Luciferase assay in hana3A cells

The Dual-Glo Luciferase Assay (Promega) was used to determine the activities of firefly and Renilla luciferase in hana3A cells (Zhuang and Matsunami, 2008). Hana3A cells were plated on poly-D-lysine-coated 96-well plates (Nalge Nunc) and incubated overnight in minimum essential medium eagle (Sigma) with 10% FBS at 37 °C and 5% CO_2. The following day, cells were transfected using Lipofectamine 2000 (Invitrogen). For each 96-well plate, 1 μg pRL-SV40, 1 μg CRE-Luc, 1 μg mouse RTP1s, and 6 μg of receptor plasmid DNA were transfected. After transfection (24 h), medium was replaced with 100 μl of odorant solution diluted in CD293 chemically defined medium (Invitrogen), and cells were further incubated for 4 h. The manufacturer's protocols were followed to measure firefly luciferase and Renilla luciferase activities. A Wallac Victor 1420 plate reader (Perkin-Elmer) was used to measure luminescence. Normalized activity was further calculated using the following formula: [Luc/RLuc(N)-Luc/RLuc(lowest)]/[Luc/RLuc(highest)-Luc/RLuc(lowest)], where Luc/RLuc(N) = luminescence of firefly luciferase divided by luminescence of Renilla luciferase in a certain well; Luc/RLuc(lowest) = lowest firefly luminescence divided by Renilla luminescence of a plate or set of plates; Luc/RLuc(highest) = highest firefly luminescence divided by Renilla luminescence of a plate. Data were analyzed using Microsoft Excel and GraphPad Prism.

Calcium imaging on the intact olfactory epithelium

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 (Protocol # 2008-001). Adult C57BL/6 mice at 2–4 months of age were used in the experiments. The method for calcium imaging on the intact olfactory epithelium was described previously (Yu et al., 2014). Briefly, the decapitated mouse head was opened along the midline, and the endoturbinates were exposed by peeling off the septum. After further removal of the olfactory bulb and bones around ectoturbinates, the whole turbinates were incubated in 10 μM fura-2 AM containing 0.02% Pluronic F-127 at 37 °C for 70 min. The turbinates were then mounted to a recording chamber with endoturbinates facing up, and were continuously perfused with oxygenated saline throughout the experiments. Ratiometric calcium imaging recording was performed as described in single cell calcium imaging.

Immunostaining

Cells were fixed with freshly prepared 2% paraformaldehyde, and then blocked by 5% albumin in PBS. Antibodies were diluted into blocking solution. Antibodies against Rhodopsin was diluted as 1:100 (a gift from Dr. Matsunami), and rabbit anti connexin 43 was diluted as 1:500 (Invitrogen). Cells were incubated with primary antibodies overnight at 4 °C, and then incubated with secondary antibodies linked to FITC and rhodamine for 1 hour at room temperature. After mounting, a confocal laser-scanning microscope (Zeiss LSM 510) was used to examine immunofluorescence.

Western blotting

2 μg cell lysates were loaded on each lane of 10% polyacrylamide gel, and then blotted onto a polyvinylidene difluoride (PVDF) membrane. After blocking with a PBST containing 5% nonfat dry milk, the blots were incubated with antibodies against phospho-connexin 43 (Ser368), ERK1/2, phospho-ERK1/2, phospho-SAPK/JNK, phospho-p38 MAPK (Cell Signaling Technologies, Beverly, MA), and connexin 43 (Invitrogen). Peroxidase-linked anti rabbit IgG (Invitrogen) were used as secondary antibodies. These proteins were visualized by using an ECL western blotting detection kit (Amersham Biosciences).