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T-type Ca^{2+} channels contribute to IBMX/forskolin- and K^+-induced Ca^{2+} transients in porcine olfactory receptor neurons

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Abstract

T-type Ca^{2+} channels are low-voltage-activated Ca^{2+} channels that control Ca^{2+} entry in excitable cells during small depolarization above resting potentials. Using Ca^{2+} imaging with a laser scanning confocal microscope we investigated the involvement of T-type Ca^{2+} channels in IBMX/forskolin- and sparingly elevated extracellular K^+-induced Ca^{2+} transients in freshly isolated porcine olfactory receptor neurons (ORNs). In the presence of mibefradil (10 μM) or Ni^{2+} (100 μM), the selective T-type Ca^{2+} channel inhibitors, IBMX/forskolin-induced Ca^{2+} transients in the soma were either strongly (>60%) inhibited or abolished completely. However, the Ca^{2+} transients in the knob were only partially (<60%) inhibited. Ca^{2+} transients induced by 30 mM K^+ were also partially (~60%) inhibited at both the knob and soma. Furthermore, ORNs responded to as little as a 2.5 mM increase in the extracellular K^+ concentration (7.5 mM K^+), and such responses were completely inhibited by mibefradil or Ni^{2+}. These results reveal functional expression of T-type Ca^{2+} channels in porcine ORNs, and suggest a role for these channels in the spread Ca^{2+} transients from the knob to the soma during activation of the cAMP cascade following odorant binding to G-protein-coupled receptors on the cilia/knob of ORNs.

Keywords: Olfaction; Signal transduction; Ca^{2+} dynamics; cAMP; Mibefradil; Ni^{2+}; Pig

1. Introduction

In most mammalian species, odor signals play critical roles in several vital functions such as feeding, reproduction, and social organization. These behaviors require the fundamental operation of odor detection and discrimination. Olfactory receptor neurons (ORNs), the sensory neurons present in the nasal cavity, are primarily involved in coding sensory information from odorous stimuli. An understanding of the mechanism of signal transduction in ORNs is thus fundamental for an appreciation of how odor signals affect animal behavior.

Based on the evidence available so far, cyclic AMP is known as the universal second messenger of olfactory signal transduction in vertebrates (Schild and Restrepo, 1998; Chen et al., 2000; Takeuchi and Kurahashi, 2003; Delay and Restrepo, 2004; Madrid et al., 2005). Other possible messengers such as $InsP_3$, NO, and CO are now believed to play modulatory roles for some odorants (Schild and Restrepo, 1998; Gold, 1999; Chen et al., 2000; Takeuchi and Kurahashi, 2003). According to the cAMP-mediated signal transduction cascade, odorant binding to G-protein-coupled receptors stimulates an adenylyl cyclase (type III), and the resulting increase in cAMP directly activates cyclic nucleotide-gated (CNG) channels, leading to an influx of Ca^{2+} (Schild and Restrepo, 1998; Ronnett and Moon, 2002). The subsequent rise in the Ca^{2+} concentration activates Ca^{2+}-dependent Cl^- conductance, giving depolarizing receptor potential during excitatory responses (Kurahashi and Yau, 1993; Lowe and Gold, 1993; Reuter et al., 1998; Reisert et al., 2003) or Ca^{2+}-dependent K^+ conductance, giving hyperpolarizing receptor

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potential during inhibitory responses (Delgado et al., 2003; Delay and Restrepo, 2004; Madrid et al., 2005). Notably, all of these major transduction events are localized to the cilia and knob, the apical portion of the dendrite exposed to the mucus layer in the nasal cavity (Menco et al., 1992; Menco, 1997; Lowe and Gold, 1991; Schild et al., 1994; Hallani et al., 1998; Kaur et al., 2001; Morales et al., 1997; Gautam et al., 2006). The signal generated at the cilia/knob has to propagate to the soma, the site of action potential generation located deep within the olfactory epithelium, for the transmission of the sensory information from odorous stimuli to the second-order neurons in the specific glomeruli in the olfactory bulb (Narusuye et al., 2003; Lowe, 2003). However, the mechanisms underlying the propagation of chemoelectrical signals from the cilia/knob to the soma remain unclear.

Experiments carried out in the last decade have also indicated that Ca^{2+} is the sole 'third messenger' of olfactory signal transduction regardless of the possible involvement of multiple second messengers (Schild and Restrepo, 1998). However, the role of Ca^{2+} in ORNs is not limited to being the third messenger of signal transduction mediating receptor potential generation, it is, rather involved in the entire sensory process taking place from the cilia to the soma, including negative feedback actions on various stages of the odor transduction mechanism and initiation of the repolarization process (by activating Ca^{2+}-dependent K^+ channels) after the generation of action potentials (Menini, 1999; Matthews and Reisert, 2003). Consistent with the complex actions of Ca^{2+}, it has been suggested that Ca^{2+} is tightly regulated in a compartmentalized manner within ORNs (Restrepo et al., 1993; Schild et al., 1994; Tareilus et al., 1995; Leinders-Zufall et al., 1997, 1998; Gautam et al., 2006). However, the mechanism underlying the spread of Ca^{2+} transients from the cilia/knob to the soma is not fully understood. So far, based on the findings from a number of vertebrate species, it is known that Ca^{2+} transients in the soma involve activation of high-voltage-activated (HVA) Ca^{2+} channels (Schild and Restrepo, 1998). In salamander ORNs it has also been reported that store-operated Ca^{2+} release in the soma may amplify the Ca^{2+} transients primarily evoked by the activation of HVA Ca^{2+} channels (Zufall et al., 2000). In a recent report, we have shown in rat ORNs that the spread of Ca^{2+} transients from the cilia/knob to the soma can also be a function of the intensity of stimulation (Gautam et al., 2006). Based on the electrophysiological properties low-voltage-activated (LVA) (T-type) Ca^{2+} channels have been reported in newt ORNs (Kawai et al., 1996; Kawai and Miyachi, 2001). However, no information is yet available about their localization or on how the presence of T-type Ca^{2+} channels may influence the spatiotemporal dynamics of Ca^{2+} during activation of the signal transduction cascade. The relevance of T-type Ca^{2+} channels in the ORNs of mammalian species also remains to be investigated.

In the present study, using a combination of IBMX (a phosphodiesterase inhibitor) and forskolin (an adenylyl cyclase activator) as the pharmacological activator of the cAMP cascade, and mibefradil and Ni^{2+} as selective inhibitors of T-type Ca^{2+} channels (Perez-Reyes, 2003), we herein show how T-type Ca^{2+} channels contribute to the spread of Ca^{2+} transients from the knob to the soma during activation of the cAMP cascade in isolated pig ORNs. Additionally, the data from the elevated extracellular K^+-induced responses are shown to facilitate understanding of the role of T-type Ca^{2+} channels during activation of the cAMP cascade by IBMX/forskolin.

2. Materials and methods

2.1. Isolation of porcine ORNs

All protocols for experiments on animals described in this work were approved by the Animal Research Committee of the Graduate School of Veterinary Medicine, Hokkaido University. Crossbred male piglets (*Sus scrofa domestica*, LW, 4–6 weeks after birth, 8–12 kg) purchased from Nishihara Farm (Rusutsu, Hokkaido, Japan) were housed with food and water ad libitum at room temperature (20–26 °C) for 2–4 days before use. Piglets were deeply anaesthetized with pentobarbital sodium (30 mg/kg, i.v., Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) after sedation with a combination of midazolam (0.5 mg/kg, i.m., Dormicum, Astellas, Tokyo, Japan) and ketamine (10 mg/kg, i.m., Ketalar, Sankyo, Tokyo, Japan); and then sacrificed by exsanguination from the carotid arteries and jugular veins. All efforts were made to minimize animal suffering. The head was quickly separated from the torso, de-skinned and sawed sagittally into two halves to expose the nasal cavity. The nasal septum and turbinates were quickly removed and transferred to ice-cold divalent cation-free Ringer's solution. Olfactory epithelium was dissected out of the underlying septal and turbinate bone/cartilage followed by mincing with dissecting scissors (1–2 mm^3) and then digested with 10 U per ml papain in divalent cation-free Ringer's solution for 10 min at room temperature. The tissue was then transferred to normal Ringer's solution, washed several times and gently triturated with a disposable plastic Pasteur pipette to dissociate the cells. The cell suspension was filtered through a nylon mesh, centrifuged at 60 × *g* for 5 min and resuspended in 1–2 ml of normal Ringer's solution. The data presented in this study were obtained from a total of 34 isolations (one piglet per isolation). In general, responses from 3 to 4 cells were recorded per isolation and each experiment employed cells from multiple isolations.

2.2. Dye loading

Twelve microliters of 1 mM Fluo-4 AM (Molecular Probes, Eugene, OR, USA) in DMSO was mixed with 990 μl of normal Ringer's solution by sonication for 40–50 s. Then 1 ml of the cell suspension was mixed with the Fluo-4 solution to give a final concentration of 6 μM Fluo-4 AM and loading proceeded for 1 h at room temperature. The cell suspension was then gently centrifuged, thoroughly washed and resuspended in 2 ml of normal Ringer's solution.

To begin the experiment, 100 μl of the suspension of Fluo-4-loaded cells was transferred to Cell-Tak (Becton Dickinson Labware, Bedford, MA, USA)-coated coverslips that were attached to the bottoms of recording chambers (volume 100 μl) with odourless dental wax. The chambers containing the cell suspension were left undisturbed for about 10 min and centrifuged at 60 × *g* for 5 min. This method was sufficient to ensure firm attachment of the dendritic knob and soma of each isolated ORN onto the coverslip of the chamber.

2.3. Calcium imaging

We recorded changes in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, using a confocal laser scanning imaging system (Fluoview FV500, Olympus, Tokyo, Japan), which allowed recording of the stimulus-induced spatiotemporal dynamics of Ca^{2+} transients in individual ORNs. The recording chamber was set on the stage of an inverted microscope with a perfusion system and the cells were continuously perfused with normal Ringer's solution at a flow rate