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# Two different avian cold-sensitive sensory neurons: Transient receptor potential melastatin 8 (TRPM8)-dependent and -independent activation mechanisms

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ABSTRACT

Sensing the ambient temperature is an important function for survival in animals. Some TRP channels play important roles as detectors of temperature and irritating chemicals. There are functional differences of TRP channels among species. TRPM8 in mammals is activated by cooling compounds and cold temperature, but less information is available on the functional role of TRPM8 in avian species. Here we investigated the pharmacological properties and thermal sensitivities of chicken TRPM8 (cTRPM8) and cold-sensitive mechanisms in avian sensory neurons. In heterologously expressed cTRPM8, menthol and its derivative, WS-12 elicited $[Ca^{2+}]_i$ increases, but icilin did not. In chicken sensory neurons, icilin increased $[Ca^{2+}]_i$, in a TRPA1-dependent manner. Icilin selectively stimulated heterologously expressed chicken TRPA1 (cTRPA1). Similar to mammalian orthologue, cTRPM8 was activated by cold. Both heterologous and endogenous expressed cTRPM8 were sensitive to mammalian TRPM8 antagonists. There are two types of cold-sensitive cells regarding menthol sensitivity in chicken sensory neurons. The temperature threshold of menthol-insensitive neurons was significantly lower than that of menthol-sensitive ones. The population of menthol-insensitive neurons was large in chicken but almost little in mammals. The cold-induced $[Ca^{2+}]_i$ increases were not abolished by the external $Ca^{2+}$ removal or by blockades of PLC-$IP_3$ pathways and ryanodine channels. The cold stimulation failed to evoke $[Ca^{2+}]_i$ increases after intracellular $Ca^{2+}$ store-depletion. These results indicate that cTRPM8 acts as a cold-sensor similar to mammals. It is noteworthy that TRPM8-independent cold-sensitive neurons are abundant in chicken sensory neurons. Our results suggest that most of the cold-induced $[Ca^{2+}]_i$ increases are mediated via $Ca^{2+}$ release from intracellular stores and that these mechanisms may be specific to avian species.



## 1. Introduction

Temperature is a critical environmental factor in homeostasis and sensing it is one of the important functions for survival and adaptation to the environment in animals (Young et al., 1989). For example, noxious heat and cold are highly unfavorable sensations that trigger powerful escape reactions in most animals. These thermosensations are involved in the regulation of physiological body temperature in mammals (Bandell et al., 2007). Mammals sense the ambient temperature through primary afferent sensory neurons of the dorsal root ganglia (DRG) or trigeminal ganglia (TG), primarily small diameter neurons. In a variety of animal species, the sensing of the ambient temperature is performed through various

*Abbreviations:* 2APB, 2-Aminoethoxydiphenyl borate; 4AP, 4-Aminopyridine; AITC, Allylisothiocyanate; AMTB, N-(3-aminopropyl)-2-{[(3-methylphenyl) methyl] oxy}-N-(2-thienylmethyl) benzamide hydrochloride salt; BCTC, 4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide; Chicken DRG, (cDRG); Chicken TRPM8, (cTRPM8); Chicken TRPA1, (cTRPA1); DRG, Dorsal root ganglion; ED, Embryonic day; PD, Postnatal day; Mouse DRG, (mDRG); Mouse TRPM8, (mTRPM8); RR, Ruthenium red; TG, Thapsigargin; TRPA1, Transient receptor potential ankyrin 1; TRPM8, Transient receptor potential melastatin 8; TRPV1, Transient receptor potential vanilloid 1; WS-12, 2-Isopropyl-5-methyl-cyclohexanecarboxylic acid (4 methoxy-phenyl)-amide; Xest, Xestospongin C.

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mechanisms (Saito and Tominaga, 2015), including a subset of temperature-sensitive transient receptor potential (TRP) channels that are called "thermoTRP" channels (Patapoutian et al., 2003). Comparison of the functions of thermoTRP channels among various species may also help to elucidate how specific channels are activated by temperature changes.

Most thermoTRP channels are calcium-permeable nonselective cation channels and are not only activated by thermal stimuli but also by other physical and chemical stimuli (Laing and Dhaka, 2016). Their activation leads to an influx of $Ca^{2+}$ and $Na^{+}$ into the cells and triggers downstream signal transduction (Bourinet et al., 2014). ThermoTRP channels have been characterized in a variety of animal species and are functionally diverse among species. For example, TRPV3 is known to be activated by warm temperatures in mammals (Xu et al., 2002), whereas it is activated by cold temperatures in amphibians (Saito et al., 2011). TRPA1 has been determined to be a putative sensor for noxious cold in mice (Story et al., 2003; Bandell et al., 2004). However, the temperature sensitivity of TRPA1 in mammals is a matter of debate. For example, primate TRPA1 is non-temperature sensitive, but rodent TRPA1 is cold sensitive (Chen et al., 2013). On the other hand, human TRPA1 expressed in artificial membranes is intrinsically cold sensitive (Moparthi et al., 2014). TRPA1 has been estimated as not only a cold sensor but also a heat sensor along with laws of thermodynamics (Clapham and Miller, 2011). A recent report using purified human TRPA1 inserted into lipid bilayer show that human TRPA1 is heat-sensitive (Moparthi et al., 2016). Amphibian and reptile TRPA1s are heat sensitive (Saito et al., 2012). Thus these species differences may be due to the difference in the thermoregulatory mechanisms of body temperature between homeotherms and poikilotherms. Recently, we characterized chicken TRPA1 (cTRPA1) and found that the channel functions as a heat sensor (Saito et al., 2014), and possesses unique chemical sensitivity compared to mammalian TRPA1 (Banzawa et al., 2014).

TRPM8 has been proposed to detect cold temperatures in the innocuous and noxious ranges of temperature (McKemy et al., 2002; Peier et al., 2002). This channel is also activated by cooling mimetic compounds such as menthol and the super-cooling agent icilin (McKemy, 2007). It has been reported that TRPM8 is mainly expressed in peripheral sensory pathways in mammals (McKemy et al., 2002; Story et al., 2003; Bautista et al., 2007). However, the functional role and pharmacological properties of chicken TRPM8 (cTRPM8) have not been fully understood. In addition, the cellular and molecular mechanisms of cold sensing in avian species are not well understood. As noted above, TRPA1, which potentially acts as a cold-sensor in mammals, is sensitive to heat in avian species, suggesting physiological roles of orthologues channels might differ among species (Saito et al., 2014). Therefore, investigation of cold sensing neurons of chicken may reveal novel molecular mechanisms which have not been recognized yet.

In the present study, therefore, we investigated the thermal sensitivity and pharmacological properties, using several agonists and antagonists that are effective on mammalian TRPM8, on heterologously and endogenously expressing cTRPM8. We also characterize the cold-sensing mechanisms in chicken DRG (cDRG) neurons. To analyze the channel activity, we used fura-2-based $[Ca^{2+}]_i$-imaging techniques with a series of pharmacological interventions since most TRP channels are highly $Ca^{2+}$ permeable (Bourinet et al., 2014).

The present results indicate that menthol and its related compound WS-12 excite chicken sensory neurons via the activation of cTRPM8. While, icilin selectively activated cTRPA1. Similar to mammals' orthologues, cTRPM8 functions as a cold-sensitive channel. In addition, we found the presence of a cold-sensing mechanism that is independent of TRPM8 in chicken. Interestingly, in cDRG neurons, menthol-insensitive cold-sensitive neurons were abundant and these $[Ca^{2+}]_i$ responses were mediated via $Ca^{2+}$ release from intracellular stores in a manner distinct from mammals. We suggest that these mechanisms are specific for temperature regulation in avian species.

## 2. Materials and methods

### 2.1. Isolation of dorsal root ganglion neurons

All procedures involved in the care and use of animals were approved by the committee on Animal Experimentation of Tottori University. All efforts were made to minimize the number of animals used. Fertilized chicken eggs were incubated (37 °C) until they reached the desired stages (embryonic day 17 to postnatal day 1; ED 17-PD 1). Adult chickens were obtained from a poultry farm in Tottori, Japan. DRG neurons were obtained from chicken according to the procedure reported previously (Banzawa et al., 2014). In brief, neonatal chick or embryo was sacrificed by decapitation. In some experiments, we used TRPA1 gene-deficient mice (TRPA1 (−/−), kindly provided by Dr. David Julius, University of California-San Francisco). $CO_2$ gas was used for euthanization for adult chickens and TRPA1 (−/−) mice. The lumbar DRGs were removed and enzymatically digested for 30 min at 37 °C with collagenase (1 mg/ml, type I, Worthington Biochem, NJ, USA) and DNase I (1 mg/ml, Roche Molecular Biochemicals, IN, USA). Subsequently, the ganglia were further treated with trypsin (5 mg/ml, Sigma-Aldrich, MO, USA) and DNase I (1 mg/ml) for 15 min at 37 °C. After enzyme digestion, the ganglia were washed with culture medium (Dulbecco's-modified Eagle's medium [DMEM, Sigma-Aldrich] supplemented with 10% fetal bovine serum [Sigma-Aldrich]), penicillin G (100 U/ml) and streptomycin (100 μg/ml). DRG cells were obtained by gentle trituration with a fire-polished Pasteur pipette. Then the cell suspension was centrifuged (800 rpm, 2 min, room temperature) and the pellet-containing cells were resuspended with the culture medium. Aliquots were placed on glass coverslips coated with poly-D, L-lysin (Sigma-Aldrich) and cultured in a humidified atmosphere of 95% air and 5% $CO_2$ at 32 °C for cDRG neurons and 37 °C for TRPA1 (−/−) mDRG neurons. In the present experiment, cells cultured within 24 h were used.

### 2.2. Expression vectors

We used expression vectors of cTRPM8 (kindly provided by Dr. David Julius), cTRPA1 and mTRPM8 that were designed to express in human embryonic kidney (HEK) 293 cells as described previously (Saito et al., 2012). HEK293 cells were transfected with expression vectors by using a transfection regent (Lipofectamine, 2000; Invitrogen, Japan). The transfected cells were incubated at 37 °C. The cells were used for $Ca^{2+}$ imaging after incubation lasting 24 h.

### 2.3. Calcium imaging

Intracellular $Ca^{2+}$ imaging of individual cells was performed with the fluorescent $Ca^{2+}$ indicator fura-2 by dual excitation using a fluorescent-imaging system controlling illumination and acquisition (Aqua Cosmos, Hamamatsu Photonics, Japan) as described previously (Ohta et al., 2008). To load fura-2, the loading temperature was set at 32 °C for cTRPA1 expressed in HEK293 cells and cDRG neurons to avoid heat activation (Saito et al., 2014). For cells expressing cTRPM8 and mTRPM8, and mDRG neurons, the loading temperature was set at 37 °C. The cells were incubated for 40 min with fura-2 acetoxymethyl ester (fura-2 AM, 10 μM, Invitrogen) in HEPES-buffered solution (in mM; 134 NaCl, 6 KCl, 1.2