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Missense mutations in Otopetrin 1 affect subcellular localization and inhibition of purinergic signaling in vestibular supporting cells

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ABSTRACT

Otopetrin 1 (*Otop1*) encodes a protein that is essential for the development of otoconia. Otoconia are the extracellular calcium carbonate containing crystals that are important for vestibular mechanosensory transduction of linear motion and gravity. There are two mutant alleles of *Otop1* in mice, *titled* (*tlt*) and *mergulhador* (*mlh*), which result in non-syndromic otoconia agenesis and a consequent balance defect. Biochemically, Otop1 has been shown to modulate purinergic control of intracellular calcium in vestibular supporting cells, which could be one of the mechanisms by which Otop1 participates in the mineralization of otoconia. To understand how *tlt* and *mlh* mutations affect the biochemical function of Otop1, we examined the purinergic response of COS7 cells expressing mutant Otop1 proteins, and dissociated sensory epithelial cells from *tlt* and *mlh* mice. We also examined the subcellular localization of Otop1 in whole sensory epithelia from *tlt* and *mlh* mice. Here we show that *tlt* and *mlh* mutations uncouple Otop1 from inhibition of P2Y receptor function. Although the *in vitro* biochemical function of the Otop1 mutant proteins is normal, *in vivo* they behave as null alleles. We show that in supporting cells the apical membrane localization of the mutant Otop1 proteins is lost. These data suggest that the *tlt* and *mlh* mutations primarily affect the localization of Otop1, which interferes with its ability to interact with other proteins that are important for its cellular and biochemical function.

Introduction

Otopetrin 1 (*Otop1*) is the founding member of a gene family that is conserved in all vertebrates (Hughes et al., 2008; Hurle et al., 2003). Otop1 is predicted to be a twelve transmembrane (TM) domain protein, containing three phylogenetically conserved Otopetrin Domains (ODI, ODII, and ODIII) (Hughes et al., 2008). ODs lack homology to any known functional domain, but retain homology to sequences found in arthropods and nematodes (Hughes et al., 2008). A function of Otop1 was first revealed by studying the phenotype of mice containing two different missense mutations in the *Otop1* gene leading to non-conservative amino acid substitutions (Hurle et al., 2003). The first mutation, *tilted* (*tlt*), is a spontaneous mutation (Ala_{151}->Glu in TM3) which is maintained on the C57BL/6J background (Lane, 1986; Ornitz et al., 1998). *Mergulhador* (*mlh*) is a mutation in *Otop1* (Leu_{408}->Gln in TM9) that arose in an ENU mutagenesis screen in a BALB/cJ genetic background (Massironi et al., 1994). Both *tlt* and *mlh* homozygous mice lack a perception of gravity and linear motion due to non-syndromic agenesis of both utricular and saccular otoconia. The resulting deficits in balance have a penetrance approaching 100%.

Otoconia are calcium carbonate crystals localized as extracellular clusters above the sensory epithelia (macula) of the utricle and saccule in the inner ear. Each crystal is composed of a proteinaceous core surrounded by a shell composed of $CaCO_3$ micro-crystals in the form of calcite (Mann et al., 1983). Otoconia are on average three times denser than the fluid (endolymph) that bathe them, and this characteristic allows them to precisely enhance an organism's sensitivity to linear motion (Carlstrom et al., 1953; Grant and Best, 1987). Resulting movement of otoconia deflects the underlying stereocilia of the hair cells in the sensory epithelium, leading to depolarization of the hair cells and propagation of electrical signals to the brain.

Abbreviations: Otop1, Otopetrin 1; tlt, Tilted; mlh, Mergulhador; OD, Otopetrin domain; TM, transmembrane domain; $[Ca^{2+}]_i$, intracellular calcium concentration; ZO-1, Zona Ocluden-1.

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Recent studies suggest a function for Otop1 in the calcification of otoconia. Otop1 expression in heterologous cells (Hughes et al., 2007) and targeted inactivation of endogenous Otop1 in mouse utricular epithelial cultures (Kim et al., 2010) demonstrate a role for Otop1 in the modulation of purinergic (P2) signaling mediated by ligands such as ATP. In the presence of Otop1, P2Y receptor-mediated Ca^{2+} release from intracellular stores was inhibited, whereas P2X receptor-like influx of Ca^{2+} was induced. These studies showed that Otop1 can modulate intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) both *in vitro* and *in vivo*. Importantly, the kinetics and characteristic of modulation of $[Ca^{2+}]_i$ by Otop1 resembled ATP-mediated increases in intravesicular $[Ca^{2+}]$ observed in isolated globular substance vesicles (putative precursors of otoconia) (Suzuki et al., 1997). This suggested that Otop1 could be the endogenous protein that regulates globular substance vesicle $[Ca^{2+}]$ to induce calcification of otoconia.

Of primary importance to understanding how Otop1 functions in the biosynthesis of otoconia is to understand the mechanism(s) by which Otop1 function is altered or disrupted by mutations that result in failure to form otoconia. *Tlt* and *mlh* mutations, found within transmembrane domains of Otop1, are predicted to alter the hydrophobicity of the transmembrane domains, which may alter protein topology, folding, trafficking, and/or biochemical activity. In this study, we examined how *tlt* and *mlh* mutations affected subcellular localization of Otop1 and the ability of Otop1 to modulate purinergic signaling both *in vitro* and *in vivo* using a transient transfection system and mouse organ culture explants. We show that *tlt* and *mlh* mutations do not interfere with the ability of Otop1 to inhibit P2Y receptor signaling, but rather alter the subcellular localization of Otop1 in the macular epithelium. This observation emphasizes the importance of apical trafficking and/or localization of Otop1 near the apical membrane of supporting cells, and suggests that Otop1 may interact with other proteins localized at or near the apical membrane or regulate the formation or composition of vesicles (globular substance) released by the sensory supporting cells.

Results

Tlt and mlh mutations do not affect Otop1 modulation of the purinergic response in vitro

Previously, COS7 cells overexpressing *EGFP*-tagged *Otop1* (*EGFP-Otop1*) were shown to modulate changes in the concentration of intracellular calcium ($[Ca^{2+}]_i$) in response to purinergic signals, such as ATP (Hughes et al., 2007). Pharmacological analysis showed that activation of P2Y receptors, which results in the release of Ca^{2+} from IP_3-sensitive intracellular stores, is denoted by the initial peak, whereas influx of extracellular Ca^{2+} by P2X receptor activation is depicted in the following plateau phase (Hughes et al., 2007). In untransfected (WT) or *EGFP*-transfected COS7 cells, change in $[Ca^{2+}]_i$ in response to ATP was characterized by an increase in $[Ca^{2+}]_i$, forming a peak, followed by a lower but sustained increased in $[Ca^{2+}]_i$ (Fig. 1D). Consistent with previous studies, overexpression of *EGFP-Otop1* resulted in a change in how COS7 cells respond to 100 μM ATP; the initial peak in $[Ca^{2+}]_i$ was dramatically reduced and the increase in $[Ca^{2+}]_i$ resulted in a sustained plateau phase until wash (Fig. 1A). EGFP-Otop1 localizes to endoplasmic reticulum (ER), golgi, and plasma membrane (PM) in COS7 cells (data not shown), but only protein localized in the PM is thought to mediate the purinergic signaling response (Hughes et al., 2007). Interestingly, the *tlt* and *mlh* mutations did not disrupt the subcellular localization of EGFP-Otop1 (data not shown) nor the ability of EGFP-Otop1 to modulate a purinergic response (Figs. 1A–C). Statistical analysis of the calcium response showed that overexpression of either the wild type or mutant forms of *EGFP-Otop1* accounted for ~80% decrease in the initial

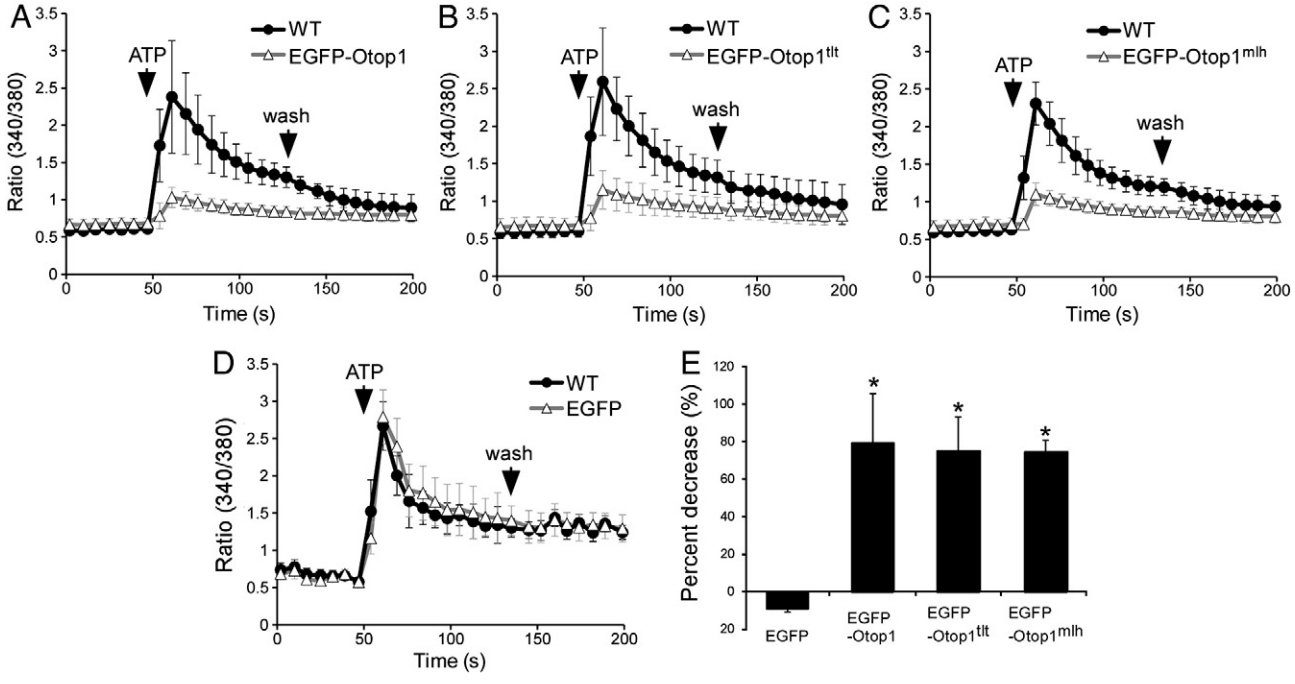

Fig. 1. Change in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in COS7 cells expressing *EGFP-Otop1*, *EGFP-Otop1^{tlt}*, and *EGFP-Otop1^{mlh}* in response to ATP. Untransfected cells are shown in black line with circle symbols and transfected cells are shown in gray line with open triangle symbols. (A) Untransfected wild type (n = 181) cells showed a biphasic response to 100 μM ATP with an initial peak followed by a plateau phase which persisted until removal of the agonist (wash). The initial peak (rise) in $[Ca^{2+}]_i$ was much lower in the presence of *EGFP-Otop1* (n = 161) where a sharp peak was absent. (B) *EGFP-Otop1^{tlt}*-positive cells (n = 190) showed a reduced initial rise in $[Ca^{2+}]_i$ compared to the wild type (n = 163) cells. (C) *EGFP-Otop1^{mlh}*-positive cells (n = 202) showed a reduced initial rise in $[Ca^{2+}]_i$ compared to the wild type cells (n = 220). (D) Transfection of EGFP (n = 7) does not alter how COS7 cells respond to ATP (wild type cells: n = 14). (E) Comparison of percent decrease of the initial peak values between untransfected and transfected cells. The percent decrease in cells transfected with *EGFP-Otop1*, *EGFP-Otop1^{tlt}*, *EGFP-Otop1^{mlh}* is significantly higher than those transfected with EGFP alone. *$p < 0.0001$ compared to EGFP control by two tailed student *t*-test. The graphs show average values ± standard deviation.