Regulation of Cellular Calcium in Vestibular Supporting Cells by Otopetrin 1

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1Departments of Developmental Biology, 2Neurology, and 3Otolaryngology, Washington University School of Medicine, St. Louis, Missouri; 4Department of Genetics, Boys Town National Research Hospital, Omaha, Nebraska; and 5Laboratory of Cell Structure and Dynamics, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, Maryland

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Kim E, Hyrc KL, Speck J, Lundberg YW, Salles FT, Kachar B, Goldberg MP, Warchol ME, Ornitz DM. Regulation of cellular calcium in vestibular supporting cells by Otopetrin 1. J Neurophysiol 104: 3439–3450, 2010. First published June 16, 2010; doi:10.1152/jn.00525.2010. Otopetrin 1 (OTOP1) is a multitransmembrane domain protein, which is essential for mineralization of otoconia, the calcium carbonate biominerals required for vestibular function, and the normal sensation of gravity. The mechanism driving mineralization of otoconia is poorly understood, but it has been proposed that supporting cells and a mechanism to maintain high concentrations of calcium are critical. Using Otop1 knockout mice and a utricular epithelial organ culture system, we show that OTOP1 is expressed at the apex of supporting cells and functions to increase cytosolic calcium in response to purinergic agonists, such as adenosine 5’-triphosphate (ATP). This is achieved by blocking mobilization of calcium from intracellular stores in an extracellular calcium-dependent manner and by mediating influx of extracellular calcium. These data support a model in which OTOP1 acts as a sensor of the extracellular calcium concentration near supporting cells and responds to ATP in the endolymph to increase intracellular calcium levels during otoconia mineralization.

INTRODUCTION

Otoconia are calcium carbonate (CaCO₃) biominerals located in the extracellular space above the sensory epithelium (macula) of the utricle and saccule of the mammalian inner ear. These high-density crystals are required for sensation of gravity. Otoconia forms by nucleation and growth of CaCO₃ crystals around an already-formed proteinaceous core composed of calcium binding and matrix proteins (Mann et al. 1983). In mice, the maximal rate of mineralization occurs between embryonic day 15 (E15) and E16.5, and the mineral growth persists until postnatal day 7 (P7) (Anniko 1980; Lim 1973).

Nucleation of CaCO₃ crystals requires a high Ca²⁺ concentration ([Ca²⁺]), although the endolymph bathing the sensory epithelium contains very low free [Ca²⁺]. As a mechanism to maintain high [Ca²⁺], a vesicular structure, called “globular substance,” is thought to be extruded from the apical surface of the maculae in the embryonic inner ear (Suzuki et al. 1995b; Tateda et al. 1998). Treatment of globular substance vesicles with adenosine 5’-triphosphate (ATP) resulted in a five- to sixfold increase in intravesicular Ca²⁺ (Suzuki et al. 1997a). This suggests that increasing the concentration of Ca²⁺ in globular substance vesicles could mediate nucleation of CaCO₃ crystals, a process that could be regulated in vivo by ATP. The kinetics and concentration dependence of the ATP-mediated [Ca²⁺] increase was most similar to that of known purinergic P2 receptors (P2Y and P2X families). P2Y receptors are metabotropic G protein coupled receptors that mediate release of Ca²⁺ from intracellular stores (Burnstock 2007). P2X family receptors are ionotropic channels that allow an influx of extracellular Ca²⁺ (North 2002).

Tilted mice lack otoconia and show a head-tilting behavior with inability to swim (Ornitz et al. 1998). Positional cloning identified that tilted is a mutant allele of Otopetrin 1 (Otop1) (Hurle et al. 2003). Otop1 is predicted to have 12 transmembrane domains and three evolutionarily conserved domains (Hughes et al. 2008). The primary structure of OTOP1 and positive expression in the utricle and saccule raised a possibility that OTOP1 may regulate Ca²⁺ influx in the globular substance vesicles and/or inner ear cells. Indeed, our previous studies demonstrated that in vitro OTOP1 is sufficient to modulate a purinergic response (Hughes et al. 2007). Overexpression of Otop1 in immortalized cell lines leads to a nonspecific depletion of endoplasmic reticulum Ca²⁺ stores, a specific inhibition of P2Y receptor signaling, and influx of extracellular Ca²⁺. However, the lack of in vivo data, which can functionally link these biochemical activities of OTOP1 with Ca²⁺ regulation in the maculae, has yet to be resolved.

Here, we demonstrate expression of Otop1 in the extrastriolar region of the utricle and saccule, with subcellular localization to the apical region of supporting cells. We present evidence that OTOP1 regulates intracellular calcium ion concentration ([Ca²⁺]) in vestibular supporting cells in vivo by inhibiting P2Y receptor-mediated intracellular Ca²⁺ release in an extracellular Ca²⁺-dependent manner in response to ATP. These data support a model by which OTOP1 concentrates Ca²⁺ in supporting cells to allow nucleation, growth, and maintenance of otoconia in a low Ca²⁺ environment.

METHODS

Generation of the Otop1βgal/βgal allele

The targeting construct was made using recombinering methods (Liu et al. 2003). First, about 5 kilobases (kb) upstream and downstream of the regions to target was retrieved from bacterial artificial chromosome (BAC) clone RP24-286E11 (derived from C57BL/J6 mice), which completely spanned the Otop1 gene. We designed a deletion of the last 62 bp of exon 2 after the Otop1c splice acceptor site and 2.7 kb of intron 2 and inserted the β-galactosidase (βgal) gene and the LoxP/pol-2-neo/LoxP selectable marker (6.1 kb). This configuration created an Otop1βgal/βgal fusion protein that includes 109 amino acid residues of OTOP1a.
107 amino acid residues of OTOP1b, or 41 amino acid residues of OTOP1c amino terminal coding sequence fused at amino acid residue 5 of βgal. The 5’ and 3’ regions of homology contained a total of 8.5 kb of genomic DNA. After verifying with restriction mapping and sequencing, the targeting construct was linearized and electroporated into SCC-10 embryonic stem (ES) cells, which were derived from 129X1/SvJ mice. Electroporation was carried out in the Washington University Siteman Cancer Center Murine Embryonic Stem Cell Core facility. G418-resistant clones were screened for homologous recombination by Southern blot using 5’ and 3’ probes. Two positive clones were identified and homologous recombination was verified using a 3’ Southern blot probe. ES clones were karyotyped and then microinjected into mouse blastocysts by the Washington University Mouse Genetics Core facility. Chimerism was determined by coat color and high percentage chimeric males were mated with C57BL6/J females. The subsequent F1 mice were intercrossed and the litter was examined for germline transmission of the Otop1βgal allele using Southern blotting with 5’ and 3’ probes extrinsic to the targeting vector and by genomic polymerase chain reaction (PCR) (Fig. 1, D and E). The LoxP/pol-2-neo/LoxP cassette was excised in vivo by mating to a mouse that expresses CRE recombinase in the germ line and then the male progeny was bred to C57BL6/J females to transfer the targeted allele onto a C57BL6/J genetic background. Phenotypes that we observed in this study appeared independent of genetic background. All animal studies were carried out in accordance with the guidelines and approval (protocol number 20070220) from the Washington University Animal Studies Committee.

**Southern blotting and genotyping**

Genomic DNA was extracted with phenol/chloroform and isopropanol precipitation. From each sample, 10 μg was digested with EcoRV at 37°C overnight and electrophoresed on a 1% agarose gel. Southern blots were probed with 5’ and 3’ probes, which were labeled with 32P (Prime-It II Random Primer Labeling Kit; Stratagene La Jolla, CA) and purified (MicroSpin S-200 HR columns; Amersham Biosciences, Pittsburgh, PA). 5’ and 3’ probes were made by amplification from RP24-286E11 BAC DNA with the following primers: primer 1 (5’-CAC CAC GTC TAA ACA AGC CA-3’ [forward]), primer 2 (5’-GCC ACA CAG GAC TTT CTT TT-3’ [reverse]); 3’ probe: primer 1 (5’-GAT AAT TTC ACA CTG TAA GC-3’ [forward]), primer 2 (5’-GAG ATT CCC TAC CAA TGA TT-3’ [reverse]). For genotyping PCR, mouse tail DNA was amplified with the following primers: primer 1 (5’-AGG GTT CCC ACG GTC CAC CCT ACA GCC-3’ [forward]), primer 2 (5’-TGA CAG CCT ACA GCC CAG GATG-3’ [reverse]); 3’ probe: primer 1 (5’-CCA TTC AGG CTG CGC AAC TGT-3’ [Otop1βgal reverse]). Thirty days of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension

![Diagram](http://jn.physiology.org/)

**FIG. 1.** Targeting the Otopetrin 1 (Otop1) gene. A: Otop1 spans over 28 kilobases (kb) of genomic DNA on mouse chromosome 5 and contains 7 exons. B: Otop1α coding sequence starts within exon 2 and is the major splice form of Otop1. Otop1b and 1c are transcribed from exon 1 and splice to different sites in exon 2. In the targeting vector, the β-galactosidase (βgal) gene and LoxP/pol-2-neo/LoxP cassette were inserted in frame with exon 2 after the Otop1c splice site. C: the targeted allele, Otop1βgal, created a fusion protein with the first 2 transmembrane (TM) domains of Otop1α at the N-terminal of βgal. The epitope to which OTO1 antibody was made is located within the N-terminal tail (red line, arrow). D: Southern blot after digesting tail genomic DNA with EcoRV. The wild-type allele produces a 17-kb fragment with both 5’ and 3’ probes. Targeted allele produces 8-kb (5’) and 9-kb (3’) restriction fragments, respectively. E: polymerase chain reaction (PCR) genotyping with tail genomic DNA. Wild-type allele, 545 bp. Targeted allele, 369 bp. F–H: histological sections through the utricle and saccule. Otoconia are normally formed above the sensory epithelia (SE) of Otop1+/+ and Otop1βgal/βgal utricle (top) and saccule (bottom) (F, G). No otoconia are observed in Otop1βgal/βgal otolithic organs (H). Scale bar: 100 μm.
at 68°C for 3 min were used to amplify either 545 (Ottop1<sup>+/−</sup>) or 369 (Ottop1<sup>+/+</sup>) base-pair (bp) fragments.

**H&E and X-gal staining**

Temporal bones were dissected in cold phosphate-buffered saline (PBS: 3.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM KCl, 135 mM NaCl, pH 7.4) and fixed in 4% paraformaldehyde at 4°C overnight. After washing in PBS, tissues were embedded in paraffin. Sections (8 μm) were deparaffinized and stained with hematoxylin and eosin (H&E). For X-gal staining, temporal bones were fixed at 4°C overnight in 0.2% glutaraldehyde, 5 mM EGTA (pH 7.3), and 100 mM CaCl<sub>2</sub> in PBS. Adult temporal bones were decalcified in 0.35 M EDTA in 0.1 M sodium phosphate buffer (pH 7.2–7.4) before further processing. For frozen sections, fixed temporal bones were incubated in 30% sucrose overnight and then frozen in OCT (optimal cutting temperature frozen tissue matrix) prior to cryosectioning. Whole-mount immunohistochemistry was performed on the frozen sections. For detection of purinergic receptors, a plate of mouse utricular maculae was dissected from postnatal day 0 (P0) to P4 rats and attached to coverslips previously coated with Cell-Tak (150 μg/μL; BD Biosciences, San Jose, CA). Cultures were maintained in DMEM/F12 (Invitrogen) with 5–7% fetal bovine serum (FBS) and ampicillin (1.5 μg/mL; Sigma) and kept at 37°C and 5% CO<sub>2</sub>. For transfections, 25 μg of 1 μM gold particles and loaded into Helios Gene Gun cartridges (BioRad, Hercules, CA). Tissue explants were transfected with the gene gun set at 95 psi of helium and maintained in culture for 24 h.

**Production of anti-Otop1 antibody**

A rabbit polyclonal antibody was made using a 16-amino acid peptide epitope (ARGSPQASGPRRGASV) derived from the N-terminus of Otop1 (Fig. 1C). The synthetic peptide was conjugated to maleimide-activated keyhole limpet hemocyanin prior to injection into the rabbit. The resulting antibody was purified on the peptide affinity column and validated by Western blotting of extracts from inner ear tissues. Further validation was performed in this study by comparing the immunostaining patterns with those of X-gal staining.

**Whole-mount immunohistochemistry**

Temporal bones were isolated in Leibovitz’s medium L15 (Sigma, St. Louis, MO) and fixed in 4% paraformaldehyde for 1–2 h at RT. Utricular and sacular maculae were dissected from the temporal bones in cold PBS, incubated in 0.5% Triton X-100 for 30 min at RT, and then washed with PBS. Samples were blocked using 4% bovine serum albumin (BSA)/PBS overnight at 4°C and incubated with rabbit anti-Otop1 (1:800), mouse anti-parvalbumin (1:500; Sigma), rabbit anti-myosin VIIa (1:500; Proteus Biosciences, Romana, CA), rabbit anti-βTectorin (1:500; a gift from Dr. Guy Richardson), rabbit anti-P2Y2 receptor (1:500; Alomone Labs, Jerusalem, Israel, a gift from Dr. Tom Steinberg), or rabbit anti-P2Y4 receptor (1:500; a gift from Dr. Tom Steinberg) for 90 min at RT in a humidified chamber. After washing with PBS, samples were incubated with secondary antibodies, Alexa 488 anti-rabbit IgG (1:600; Invitrogen), Alexa 555 anti-mouse IgG (1:600; Invitrogen), or Cy3 anti-rabbit IgG (1:200; Chemicon, Billerica, MA), respectively, for 45 min at RT. For stereocilia staining, samples were further incubated with rhodamine-phalloidin (1:200; Invitrogen) for 15 min at RT. After washing with PBS, samples were transferred to a superfrost slide, mounted with Vectashield (Vector Labs, Burlingame, CA), and coverslipped.

**Overexpression of EGFP-Otop1 in utricular culture cells using gene gun transfection**

Pieces of the utricular maculae were dissected from postnatal day 0 (P0) to P4 rats and attached to coverslips previously coated with Cell-Tak (150 μg/μL; BD Biosciences, San Jose, CA). Cultures were maintained in DMEM/F12 (Invitrogen) with 5–7% fetal bovine serum (FBS) and ampicillin (1.5 μg/mL; Sigma) and kept at 37°C and 5% CO<sub>2</sub>. For transfections, 50 μg of EGFP-Otop1 was precipitated onto 25 ng of 1 μM gold particles and loaded into Helios Gene Gun cartridges (BioRad, Hercules, CA). Tissue explants were transfected with the gene gun set at 95 psi of helium and maintained in culture for 24 h.

**Utricular macular organotypic and dissociated cultures**

E18.5–P3 mouse utricles were dissected in Medium 199 (Gibco #12350039) and the nonsensory epithelium and otocional layer were completely removed. After incubation in thermolysin (25 mg in 50 ml Medium 1999; Gibco #12340030; Sigma) at 37°C for 50 min, the underlying stroma was removed and the remaining epithelial sheet was attached to a MatTek dish using extracellular matrix (ECM) gel (Sigma). The tissue was incubated in Medium 199 (Gibco #12340030) containing 10% FBS (Gibco #10091-155 growth media) overnight at 37°C. For dissociated culture, isolated epithelial sheets were treated with trypsin/EDTA (0.05%, 0.02%) for 15 min at 37°C. After replacing trypsin/EDTA with Medium 199 (Gibco #12340030), cells were triturated 5 to 10 times and plated on laminin-coated MatTek dishes and incubated overnight at 37°C. The next day, growth media was added.

**Ratiometric calcium imaging and data analysis**

All imaging experiments were carried out at RT in a HEPES-buffered salt solution (HCSS) containing (in mM): 140 NaCl, 5.4 KCl, 1 NaH<sub>2</sub>PO<sub>4</sub>, 1.8 CaCl<sub>2</sub>, 1 MgSO<sub>4</sub>, 12 HEPES, and 5.5 p-glucose, pH 7.4 ± 0.1 or nominally Ca<sup>2+</sup>-free media (HCSS solution without CaCl<sub>2</sub>). Organotypic or dissociated utricular macular cultures were loaded with fura-2 by incubation for 60 min with 5–10 μM fura-2 acetoxyethyl (AM) ester (Invitrogen) and 0.1% Pluronic F-127 (Invitrogen) in HCSS (pH = 7.2) at RT, washed with HCSS, and incubated for another 60 min to allow for ester hydrolysis. After loading, cells were imaged on an inverted microscope (Nikon Eclipse...
TE300; Nikon, Melville, NY) equipped with a cooled charge-coupled camera (Cooke, Auburn Hill, MI) using a ×200/0.45 Plan Fluor objective (Nikon). The fluorescence excitation (75 W xenon arc lamp) was provided by band-specific filters (340 and 380 nm; Semrock, Rochester, NY) in combination with an XF73 dichroic beam splitter (Omega Optical, Brattleboro, VA). Pairs of images were collected constantly at alternate excitation wavelengths. After subtracting the matching background, the image intensities were divided by one another to yield ratio values for individual regions. For studies done with mouse macular dissociated cultures (Fig. 6), 10 μM ionomicyn (EMD Biosciences, Gibbstown, NJ) was added at the end of each experiment to obtain the maximum ratio (340/380) in each region of interest. To reduce variability between experiments performed on different days, raw data were normalized as follows: Normalized Ratio = (R − R0i)/(R_max − R0), where R is the individual ratio value, R_max is the peak ratio after addition of ionomicyn, and R0 is the average of the prestimulation baseline ratio. Absolute baseline values were comparable between experiments. All data in Fig. 6, E–I represent normalized ratios. All chemicals were purchased from Sigma, unless otherwise indicated. To compare the rates of decay in intracellular [Ca2+]i, following the initial peak response to purinergic agonists (Fig. 6A), a single exponential decay curve (y = y0e−t/τ, where y is the normalized ratio, t is time and τ is the decay constant) was fitted to the normalized ratio values collected within 90 s after the initial peak. Decay constants (τ) were then averaged for each experiment and normalized to Otop1+/+ tissues treated with ATP. To compare the rates of decay in the plateau phase (Supplemental Fig. S4) we used the same methods to analyze data points, but starting 40 s after the initial peak and extending until wash.1 Significant differences between the decay constants calculated for different experimental conditions were determined by a two-tailed Student’s t-test.

RESULTS

Otopetrin 1 (Otop1) deficiency results in otoconial agenesis

To generate a functional null allele for Otop1 and to create a histochemical tag to identify OTOP1-expressing cells, the Otop1 gene was targeted by insertion of a β-galactosidase (βgal) coding sequence (Fig. 1). Otop1 has seven exons and three identified amino terminal alternative splice variants, Otop1a, 1b, and 1c (Fig. 1, A and B). Although Otop1a is the most abundant splice variant expressed in the inner ear (Hurle et al. 2003), to ensure generation of a functional null allele, the βgal cDNA was inserted in frame in exon 2 such that all known splice variants would be captured as fusion proteins with βgal. This design expressed Otop1βgal transcripts under the control of Otop1 transcriptional regulatory elements. For the Otop1dβgal and Otop1βgal transcripts, this fusion protein includes the first two TM domains of OTO1P1 (Fig. 1C). The germ-line-transmitted targeted allele was bred to homozygosity. Heterozygous Otop1βgal+ mice were phenotypically normal (Fig. 1, F and G). Otop1βgalβgal+ mice showed vestibular dysfunction (inability to swim) and otoconial agenesis (Fig. 1H). The sensory epithelium of the utricle and saccule were histologically normal.

OTOP1βgal expression in the vestibular sensory epithelium

The spatial and temporal expression pattern of OTOP1βgal in the inner ear was assessed by X-gal staining. βgal activity was first apparent at embryonic day E13.5 in the utricle (Fig. 2, A and B), whereas expression in the saccule was first detected 1 day later, at E14.5 (Fig. 2, C and D). By E16.5, the staining was present throughout the whole epithelium in the utricle and saccule (Fig. 2, E and F). This expression pattern correlated with the onset and maximal rate of otoconial synthesis in the utricle and saccule (Anniko 1980; Anniko et al. 1987). Interestingly, examination of isolated whole maculae revealed a striking absence of expression in the striolar region (Fig. 2, G and H), a pattern that persisted in the adult ears (data not shown). In addition to expression in nonstriolar regions of the macula, some transitional cells were also positive for βgal activity (Fig. 2, G and I), indicating possible involvement of a subset of transitional cells that most likely function in the calcification process during otoconial development. Importantly, no expression was observed in any other inner ear organs or structures ( cristae or cochlea) at any time point examined, with the exception that, at E18.5, trace βgal activity was occasionally detected in the endolymphatic sac (data not shown).

Histological sections through the maculae revealed a staining pattern consistent with expression in the maculae (black arrowheads) and transitional cells (white arrowheads) (Fig. 2J). Inner ear sections from P12 and 6-mo-old mice (Fig. 2, K and L) showed that Otop1 continues to be expressed in the adult inner ear. Depending on the orientation of the sections, we observed the absence of X-gal staining within the central maculae, which reflected lack of expression in the striolar region (Fig. 2, G, H, and K). To confirm expression in adult mice, endogenous Otop1 mRNA was detected by quantitative RT-PCR. Consistent with the βgal activity, Otop1 expression was detected in both P2 and 6-mo-old utricle and saccule, but not in the cochlea or lung (Fig. 2M). These data suggest that OTOP1 could function to maintain otoconia mineralization in the adult.

OTOP1 is localized toward the apex of supporting cells

Otonia are formed within the gelatinous membrane above the utricular and saccular sensory epithelia. We hypothesized that OTOP1 protein would be localized at a subcellular site consistent with a role in the biosynthesis and/or maintenance of otoconia. Polyclonal anti-OTOP1 antibody was generated to an epitope located in the amino terminus of OTOP1 and, because this epitope is maintained in the OTOP1βgal fusion protein (Fig. 1C), immunostaining of Otop1βgalβgal tissues revealed the location of the chimeric protein. Colocalization of anti-OTOP1 and anti-βgal signals on Otop1βgalβgal sections (Fig. 3, D–F) confirmed the specificity of the anti-OTOP1 antibody.

In Otop1+/- macula, endogenous OTOP1 immunoreactivity was concentrated toward the apical surface of the epithelium (Figs. 3, A–C and 4, A–C). Colocalization of OTOP1 with zona occludens 1 (ZO-1) protein showed that some OTOP1 protein is localized at the apical cell surface (Fig. 4, A–C). In Otop1βgalβgal and Otop1βgalβgal+ utricles, one or two copies of the targeted allele resulted in formation of large inclusions, with Otop1βgalβgal+ and Otop1βgalβgal++ utricle and saccule with the anti-OTOP1 antibody was used to identify cell-specific expression of the endogenous OTOP1 protein. Cosingaining with either phallodin (to mark stereocilia) (Fig. 4, D and E) or α-parval-
that this population of supporting cells is crucial for development of sensory epithelium. To examine whether deletion of Otop1 could affect development of hair cells or the striola, immunostaining with Myosin VIIa or βTectorin was performed, respectively (Supplemental Fig. S2). Normal expression and localization of these markers suggested that otoconia agenesis and lack of Otop1 in Otop1β/β inner ears is unlikely to affect hair cell development or formation of the striola.

**OTOP1 modulates purinergic response in the utricular maculae**

Previous in vitro studies (Hughes et al. 2007) identified a potential role for OTOP1 in modulating cytosolic free calcium ion concentration ([Ca2+]i) in response to purinergic signals, such as ATP. Because endolymph is known to contain ATP and purinergic receptors have been identified in several cell types within the inner ear (Housley 2000; Lee and Marcus 2008), we hypothesized that OTOP1 may regulate purinergic signaling in vestibular supporting cells. To determine whether...
endogenous OTOP1 is necessary to regulate a purinergic response in mouse utricular epithelium, organotypic utricular macular cultures from Otop1+/+ and Otop1βgal/βgal (Fig. 6, B and C). Similarly to dissociate mouse maculae resulted in cultures that contained regions of epithelial sheets and clusters of cells that formed globule-like structures and was rarely found in the adjacent epithelial sheets (Fig. 6D, arrow, asterisk). Therefore the regions near the globule-like structures were selected for imaging (Fig. 6, B and C). Similar to the response seen in organotypic cultures (Fig. 5D), addition of ATP to Otop1βgal/βgal dissociated macular cells resulted in a higher peak and increased rate of decay than that observed in Otop1+/+ dissociated cultures (Fig. 6, E and J). This result confirmed that dissociated cultures behave similarly to whole maculae and can therefore be used to further characterize the function of OTOP1. Importantly, this differential response between Otop1+/+ and Otop1βgal/βgal cultures was no longer observed when the response to ATP was compared from regions of the cultures that did not express OTOP1 (in Otop1+/+ cultures) or OTOP1βgal (in Otop1βgal/βgal cultures) (Fig. 6F), confirming the specificity of OTOP1 activity. Notably, Otop1+/+ and Otop1βgal/βgal cultures showed a similar response to ATP (Fig. 6E), demonstrating that a single Otop1 allele is sufficient for OTOP1-specific modulation of the purinergic response and is consistent with the presence of normal otoconia in Otop1βgal/+ mice (Fig. 1G).

Activation of P2Y receptors results in release of Ca2+ from inositol-1,4,5-trisphosphate (IP3)-sensitive intracellular stores, whereas the P2X receptor family acts as a channel for Ca2+ influx from the extracellular space. To determine which purinergic receptor family is regulated by OTOP1 in vivo, we sought to block P2Y or P2X-mediated Ca2+ signaling by restricting Ca2+ influx into the cytosol from intracellular stores or the extracellular space, respectively. Bisphenol inhibits the functions of intracellular Ca2+-ATPases, thus consequently emptying intracellular Ca2+ stores and uncoupling P2Y receptor signaling (Brown et al. 1994; Harper et al. 2005). In the presence of bisphenol, the response of Otop1+/+ cultures to ATP did not significantly change (Fig. 6, E and G). However, Otop1βgal/βgal cultures no longer showed the high peak after addition of ATP and responded in a similar manner with a peak and rate of decay comparable to that of Otop1+/+ cultures (Fig. 6, E, G, and J). This suggests that the high peak originally shown in Otop1βgal/βgal cultures (Fig. 6E) is indicative of P2Y-mediated Ca2+ release and that OTOP1 normally interferes with this process. Our macular epithelial cultures express all seven P2Y isoforms (Supplemen-
tal Fig. S3) and each isoform has distinct agonist sensitivity (Burnstock 2007; Ralevic and Burnstock 1998). To identify which P2Y receptor function is inhibited by OTOP1, we additionally performed ratiometric Ca²⁺/H⁺ imaging with three other major purinergic ligands: UTP, ADP, and UDP. Since the initial response after addition of the ligand indicates an increase in [Ca²⁺/H⁺]i by P2Y-mediated signaling, we looked for ligands that would result in a significant difference in the initial peak level between Otop1+/+ and Otop1βgal/βgal cultures. Treating cultures with UTP, a P2Y-specific ligand (Supplemental Fig. S4) (Burnstock 2007; Ralevic and Burnstock 1998), allowed us to examine the effect of OTOP1 exclusively on P2Y receptors and not on P2X receptors. Notably, similar to what was observed with ATP, in response to UTP, [Ca²⁺/H⁺]i in Otop1βgal/βgal cultures increased to a higher level when compared with that in Otop1+/+ cultures (Fig. 6H). However, significant differences in the peak value were not observed following treatment with either ADP or UDP (Supplemental Fig. S5). This suggested that OTOP1 might preferentially inhibit the function of P2Y receptor subtypes featuring high affinity to ATP and UTP, such as P2Y2 and P2Y4 (Dubyak 2003; Ralevic and Burnstock 1998). Other purinergic receptors expressed in the macular epithelium that are not regulated by OTOP1 may also contribute to the observed increase in [Ca²⁺/H⁺]i.
in response to ADP or UDP. Alternatively, the inhibitory function of OTO1 may require ATP or UTP but not ADP or UDP.

Next, we investigated the source of Ca²⁺ that contributes to elevated [Ca²⁺], (plateau phase) in response to ATP in the presence of OTO1. If OTO1 inhibits all P2Y-mediated Ca²⁺ release and wholly relies on extracellular Ca²⁺ for increased [Ca²⁺], in response to ATP, we would not see any increase in [Ca²⁺] in OTO1+/+ cultures in Ca²⁺-free media. In support of this model, a similar response to ATP was observed when imaging in either “nominally” Ca²⁺-free media (containing 10–20 μM Ca²⁺, determined with a Ca²⁺-selective electrode and fura-2 fluorometry; Fig. 6f) or Ca²⁺-free media (normal media with 10 mM EGTA which contains <1 nM Ca²⁺, data not shown). Notably, similar results were also obtained when the imaging was performed in nominally Ca²⁺-free media (Fig. 6f) or continuously in the same dish, first with normal media and second following a change to nominally Ca²⁺-free media (Supplemental Fig. S6). Interestingly, OTO1+/+ cultures in nominally Ca²⁺-free media did show a response to ATP with similar peak levels to that shown in the presence of extracellular Ca²⁺, but the following plateau phase was much reduced, resulting in an increased rate of decay (Fig. 6, E, I, and J). This suggests that OTO1 interferes with P2Y receptor function (possibly P2Y2 and/or P2Y4) and promotes extracellular Ca²⁺ influx. OTO1βgal/βgal cultures also showed a reduction in the plateau phase but, most interestingly, the difference in the initial peak value between OTO1+/+ and OTO1βgal/βgal cultures was no longer observed when extracellular Ca²⁺ was removed. A possible explanation for this observation is that the function of OTO1, to inhibit P2Y activity, may depend on extracellular Ca²⁺ (at concentrations higher than those in nominally Ca²⁺-free media). These data support a model in which OTO1 inhibits P2Y receptor-mediated Ca²⁺ release in macular epithelial cells in a Ca²⁺-dependent manner and promotes an influx of Ca²⁺ in response to ATP.

DISCUSSION

OT1 is essential for otoconia formation and thus for the normal physiological function of the gravity receptor organs in the inner ear. Several missense alleles in OTO1 have been identified (tilted, mergulhador, backstroke) and all lead to otoconial agenesis in the mouse or otolith agenesis in the zebrafish (Hurle et al. 2003; Sollner et al. 2004). The biochemical function(s) of OTO1 is poorly understood and the biochemical consequences of these missense mutations are not known. To interpret phenotype in relation to the biochemical function of OTO1 alleles requires knowledge of the genetic null allele and its phenotype. To this end, we have created a functional null for OTO1, tagged with β-galactosidase (OT01βgal). Homozygous OTO1βgal/βgal mice have otoconial agenesis, similar to that seen in tilted and mergulhador mice.

The OTO1βgal allele permitted a highly sensitive assay to identify spatial and temporal patterns of OTO1 expression and to validate an antibody directed against OTO1. Staining whole maculae for β-galactosidase activity revealed several novel features of OTO1 expression. Most remarkably, OTO1 is excluded from the striola in both the utricle and saccule, but is intensely expressed in the remaining regions of the maculae. Otoconia size ranges from 0.1 to 25 μm in mammals (Mann et al. 1983; Ross and Pote 1984) and the striola region of the utricle is known to have fewer and smaller otoconia. In the saccular striola, otoconia are again smaller, but are greater in number, forming a ridge in the otoconial layer (Lim 1973; Lindeman 1973). Thus otoconia formed above the site where OTO1 is expressed are likely to be larger in size in the extrastriolar areas, correlating OTO1 expression with its proposed role in the regulation of otoconia growth or calcification. We hypothesize that otoconia found above the str...
riola originated in adjacent regions expressing Otop1 and ceased growing once removed from regions above Otop1 expression. Similarly, in other sites in the inner ear that do not express Otop1 (ampulla and cochlea), otocoria are not formed even though the major otoconia core protein, Otoconin 90, is ubiquitously expressed throughout the inner ear (Verpy et al. 1999; Wang et al. 1998).

Staining histological sections of the maculae for βGAL activity and immunohistochemical detection of endogenous OTOPI revealed that OTOPI is expressed in the supporting and a subset of transitional cells of the mouse utricle and saccule, but not in hair cells. In contrast, zebrafish Otop1 mRNA was localized to hair cells during otolith growth (Sollner et al. 2004). This disparity between mouse and zebrafish
might reflect differences in the cell types that are critical for otoconia formation. Importantly, it has been shown that hair cells in mice (Bermingham et al. 1999; Hughes et al. 2006; Lundberg et al. 2006) and supporting cells in zebrafish (Haddon et al. 1999; Hughes et al. 2006; Lundberg et al. 2006) are not required for otoconia/otocyst formation.

Otoconia are formed during embryonic and perinatal development and Otop1 is expressed throughout this time. Once formed, the initial complement of otoconia is thought to last throughout the life of a mammalian organism, with only limited amounts of Ca\(^{2+}\) turnover (Balsamo et al. 2000; Preston et al. 1975). The observation that Otop1 expression persists in the adult (mice) suggests a possible role for OTOPI in the maintenance and/or repair of otoconia mineralization. Demonstration of such a role for OTOPI will require conditional inactivation of Otop1 at a time after otoconial development is complete. In humans, otoconia are prone to degeneration and displacement, leading to benign positional vertigo and progressive loss of balance (House and Honrubia 2003; Ross et al. 1976; Wellin et al. 1997). It is not known whether, in humans, Otop1 is expressed throughout life and whether OTOPI is necessary to maintain or repair otoconia. Analysis of Otop1 expression in human utricle and saccule maculae could provide a clue as to whether OTOPI has a conserved role in the maintenance of otoconia in different species. It will also be interesting to investigate whether deformation and fragmentation of otoconia, a frequent occurrence in old animals (Jang et al. 2006) and humans (Walther and Westphol 2007), results from diminished expression or activity of OTOPI.

Previous studies showed that OTOPI expression in heterologous cells could modify the cellular response to purinergic signals, resulting in altered \([Ca^{2+}]\), (Hughes et al. 2007). To determine whether a similar OTOPI-dependent biochemical activity exists in inner ear supporting cells in vivo, we examined OTOPI function in mouse utricular epithelial cultures. Comparing purinergic response in Otop1\(^{+/+}\) and Otop1\(^{balb/balb}\) mouse utricular macular epithelial cultures identified distinct functions by which OTOPI modulates purinergic signaling in vivo. It is important to note that the observed modulation of purinergic signaling caused by the absence of OTOPI could be an indirect consequence on the expression or localization of another critical protein that normally interacts with OTOPI. Importantly, these studies show that endogenous OTOPI functions to inhibit P2Y receptor activity and subsequent release of Ca\(^{2+}\) from intracellular stores in response to purinergic stimuli in vivo. This function was closely phenocopied by depleting intracellular Ca\(^{2+}\) stores by application of bisphenol, an inhibitor of all microsomal Ca\(^{2+}\)-ATPases that effectively uncouples Ca\(^{2+}\) release from P2Y signaling (Brown et al. 1994; Harper et al. 2005). Interestingly, thapsigargin (TG), which functions similarly to bisphenol by inhibiting sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase, did not have any effect on macular epithelial cultures (data not shown). Considering several previous studies that have identified TG-resistant hosts (Tanaka and Tashjian Jr 1993; Waldron et al. 1995; Watson et al. 2003), it is possible that macular epithelial cells may contain unique TG-resistant intracellular Ca\(^{2+}\) stores, possibly identifying a unique cellular compartment in these cells.

The function of P2Y signaling during otoconia formation is not known. However, recent studies have identified a role for P2Y receptors in bone mineralization, which share a number of common features with otoconia mineralization (Hughes et al. 2006; Orriss et al. 2007). In osteoblasts, P2Y2 receptor activation by ATP and UTP inhibits mineralization in vitro and P2Y2\(^{-/-}\) mice show increased bone mineral density (Hoebertz et al. 2002; Orriss et al. 2007). In the inner ear, multiple P2Y receptors are expressed in the sensory (Supplemental Fig. S3) and nonsensory epithelium (Piazza et al. 2007; Sage and Marcus 2002; Vlajkovic et al. 2007) and could function to inhibit ectopic mineralization in the inner ear. Suppressing ectopic mineralization would be essential because ectopic mineral deposits or otoconia fragments in the semicircular canals (cupulolithiasis) are thought to be a major cause of positional vertigo (Marom et al. 2009; Barnes and McClure 1992) and ectopic mineralization in the cochlea could impair auditory function. Inhibition of P2Y receptor signaling by OTOPI in the macular epithelium could serve to locally suppress P2Y activity and thus be permissive to localized mineralization of otoconia. An important long-term goal would be to produce P2Y (most preferentially P2Y2 or P2Y4) and Otop1 double-null mice to see whether the loss of P2Y function rescues the Otop1 mutant phenotype.

An additional passive mechanism that could prevent ectopic mineralization in the inner ear is the very low concentration of Ca\(^{2+}\) ions normally found in endolymph (10–100 \(\mu\)M) (Anniko 1980; Salt et al. 1989). However, a consequence of low endolymph \([Ca^{2+}]\) is the requirement to locally increase \([Ca^{2+}]\) at the site and time of otoconia nucleation and growth. This could be achieved by nucleating otoconia within Ca\(^{2+}\)-rich vesicles released from supporting cells and/or by maintaining local high concentrations of Ca\(^{2+}\) in the otoconial membrane. The presence of globular substance vesicles (Suzuki et al. 1995a, 1997b) and Ca\(^{2+}\)-ATPase activity in the apical end of the supporting cells (Yoshihara et al. 1987) support both mechanisms. The observation that endogenous OTOPI promotes a P2X receptor-like influx of extracellular Ca\(^{2+}\) suggests a possible role for OTOPI in sequestration or mineralization.
trafficking of Ca$^{2+}$ in the maculae. As a transmembrane domain protein, OTOP1 could function as a novel regulator of Ca$^{2+}$ flux, which does not share homology with any known multitransmembrane domain proteins or it may induce activity of other P2X-like channels. It is possible that OTOP1-regulated flux of Ca$^{2+}$ in supporting cells is a prerequisite for locally increasing [Ca$^{2+}$], either directly in the extracellular space of the otocional membrane or within globular substance vesicles. Notably, the characteristics of the ATP-induced increase in [Ca$^{2+}$], by OTOP1 was very similar to the atypical purinergic response described by Suzuki et al. (1997a) in isolated globular substance vesicles, suggesting that OTOP1 may be responsible for the ATP-mediated increases in intravesicular [Ca$^{2+}$], seen in these studies. Differences in the observed response to UTP may be due to the presence of different proteins in globular substance vesicles and supporting cells or due to species-specific differences. In addition to OTOP1, plasma membrane Ca$^{2+}$-ATPase 2 (PMCA2) is expressed on the apical surface of sensory hair cells (Dumont et al. 2001) and Pmca2$^{-/-}$ mice show agenesis of otoconia (Kozel et al. 1998). Notably, [Ca$^{2+}$] in lower in Deafwaddler (Pmca2 frameshift mutation) endolymph compared with controls (Wood et al. 2004). Thus both OTOP1 and PMCA2 may cooperate to regulate local concentrations of Ca$^{2+}$ above the macular epithelium.

To investigate the requirement for extracellular Ca$^{2+}$ for OTOP1 activity, wild-type and OTop$^{+/+}$otetrr BOA$^{+/+}$ macular epithelial cultures were assayed for purinergic responses in “nominally” Ca$^{2+}$-free media and Ca$^{2+}$-free media (with 10 mM EGTA). The [Ca$^{2+}$] in nominally Ca$^{2+}$-free media is similar to the [Ca$^{2+}$] in endolymph (10–20 μM). The observation that OTOP1 could no longer inhibit P2Y function in nominally Ca$^{2+}$-free media (or in the presence of 10 mM EGTA) suggests that OTOP1 is very sensitive to extracellular [Ca$^{2+}$]. In future studies, it will be important to test whether OTOP1 can bind Ca$^{2+}$ and acts as a sensor for extracellular [Ca$^{2+}$] or interact with other Ca$^{2+}$ sensor molecules to form a feedback signal to regulate [Ca$^{2+}$] above the macular sensory epithelium.

The studies presented here demonstrate that one function of OTOP1 is to modulate purinergic signaling in macular supporting cells (Fig. 7). This raises the important question of the role of ATP in endolymph. ATP is thought to be stored and released from vesicles in the marginal cells of the stria vascularis. In the vestibular system nonsensory epithelium, dark cells released from vesicles in the marginal cells of the stria vascula
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Disclosures

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