Mice Lacking Inositol 1,4,5-Trisphosphate Receptors Exhibit Dry Eye

Takaaki Inaba1,3, Chihiro Hisatsune3, Yasumasu Sasaki1, Yoko Ogawa1, Etsuko Ebisui3, Naoko Ogawa3, Minoru Matsui4, Tsutomu Takeuchi2, Katsuhiko Mikoshita3,5*, Kazuo Tsubota1

1 Department of Ophthalmology, Keio University School of Medicine, Shinjuku, Tokyo, Japan, 2 Department of Rheumatology, Keio University School of Medicine, Shinjuku, Tokyo, Japan, 3 Laboratory for Developmental Neurobiology, RIKEN Brain Science Institute, Wako, Saitama, Japan, 4 Department of Pharmacy, Chiba Institute of Science, Choshi, Chiba, Japan, 5 Calcium Oscillation Project, International Cooperative Research Project-Solution Oriented Research for Science and Technology, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan

Abstract

Tear secretion is important as it supplies water to the ocular surface and keeps eyes moist. Both the parasympathetic and sympathetic pathways contribute to tear secretion. Although intracellular Ca2+ elevation in the acinar cells of lacrimal glands is a crucial event for tear secretion in both the pathways, the Ca2+ channel, which is responsible for the Ca2+ elevation in the sympathetic pathway, has not been sufficiently analyzed. In this study, we examined tear secretion in mice lacking the inositol 1,4,5-trisphosphate receptor (IP3R) types 2 and 3 (ltp2−/−;ltp3−/− double-knockout mice). We found that tear secretion in both the parasympathetic and sympathetic pathways was abolished in ltp2−/−;ltp3−/− mice. Intracellular Ca2+ elevation in lacrimal acinar cells after acetylcholine and epinephrine stimulation was abolished in ltp2−/−;ltp3−/− mice. Consequently, ltp2−/−;ltp3−/− mice exhibited keratoconjunctival alteration and corneal epithelial barrier disruption. Inflammatory cell infiltration into the lacrimal glands and elevation of serum autoantibodies, a representative marker for Sjögren’s syndrome (SS) in humans, were also detected in older ltp2−/−;ltp3−/− mice. These results suggested that IP3Rs are essential for tear secretion in both parasympathetic and sympathetic pathways and that ltp2−/−;ltp3−/− mice could be a new dry eye mouse model with symptoms that mimic those of SS.

Introduction

Because tears keep the cornea and conjunctiva continuously moist, and a reduction in tear volume results in dry eyes (e.g. keratoconjunctivitis sicca), investigation of the regulatory mechanisms underlying tear secretion is crucial for understanding the pathology of ocular systems and for the development of new treatments for dry eyes.

Tear secretion from the lacrimal glands is regulated by two types of nerves: parasympathetic and sympathetic. The activation of parasympathetic and sympathetic nerves predominantly releases the neurotransmitters acetylcholine (Ach) and norepinephrine, respectively [1,2]. Upon binding to muscarinic acetylcholine receptors, Ach activates phospholipase C and produces inositol 1,4,5-trisphosphate (IP3), which in turn triggers intracellular Ca2+ release through the IP3 receptor (IP3R) from the endoplasmic reticulum (ER) in lacrimal gland acinar cells [1]. Stimulation of the α- and β-adrenergic receptors by norepinephrine also induces Ca2+ release from internal stores [1,2]. However, in contrast to the established role of IP3Rs in the cholinergic pathway, the Ca2+ channels that contribute to Ca2+ elevation in the sympathetic pathway are still obscure. It was reported that the activation of α1-adrenergic receptor, a predominant type of adrenergic receptor in lacrimal glands, increases intracellular Ca2+ without IP3 production, and cyclic ADP-ribose is thought to be involved in the Ca2+ increase via the ryanodine receptor—another Ca2+ channel on the ER [2–5].

To examine the physiological role of IP3Rs in the sympathetic pathway of lacrimal glands, we measured tear secretion in IP3-deficient mice (ltp2−/−;ltp3−/−), in which several exocrine secretion pathways were disrupted [6,7]. We found that ltp2−/−;ltp3−/− mice showed impaired tear secretion via both the parasympathetic and sympathetic pathways and therefore exhibit dry eye. In addition, we detected abnormalities in ltp2−/−;ltp3−/− lacrimal gland tissues, such as inflammation, infiltration, and elevated autoantibodies, and these abnormalities mimic human Sjögren’s syndrome (SS). Thus, the ltp2−/−;ltp3−/− mouse is a new dry eye animal model caused by disturbed Ca2+ signals in lacrimal glands.

Materials and Methods

Ethics Statement

All animal procedures in this study were approved by the Animal Experimental Committees at the Institutes of Physical and Chemical Research (RIKEN) –Research Center for Brain Science
Institute (BSI) (Permit Number: H25-2-202). All efforts were made to minimize animal suffering. Mice [6] were housed on a 12 h light–dark cycle, with the dark cycle occurring from 8:00 P.M. to 8:00 A.M in a specific pathogen-free environment of the Laboratory Animal Facility of the RIKEN Brain Science Institute. In all experimental groups, mice were used at 6–40 weeks of age and 50% were female. Tear collection from mouse eyes was performed under anesthesia with intraperitoneal injection of ketamine and xylazine.

**Immunoblotting**

Tissues from the lacrimal glands were homogenized in a solution containing 0.32 M sucrose, 5 mM Tris-HCl (pH 7.4), 1 mM ethylene diamine tetraacetic acid, 0.1 M phenyl methyl sulfonyl fluoride, 10 mM leupeptin, 10 mM pepstatin A, and 1 mM 2-mercaptoethanol (homogenizing buffer). The homogenate containing the lacrimal glands was centrifuged at 1000 \( g \) for 5 min at 4 \( ^\circ \)C, and the precipitated lacrimal glands were lysed with sample buffer (125 mM Tris-HCl, pH 6.8; 20% glycerol; 4.0% sodium dodecyl sulfate [SDS]; 10% 2-mercaptoethanol; 0.1% bromophenol blue). A total of 50 \( \mu \)g protein was separated by 5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to

**Figure 1. Defects in tear secretion in \( \text{Itpr}^2/\alpha - \text{Itpr}^3/\alpha \) mice via both parasympathetic and sympathetic pathways.** (A) Tear volume in wild-type (n = 12) and \( \text{Itpr}^2/\alpha \) (n = 16) mice within 15 min of pilocarpine stimulation. (B) Average body weight of wild-type and the \( \text{Itpr}^2/\alpha \) mice at 6 weeks. (C) Average lacrimal gland weights of wild-type and the \( \text{Itpr}^2/\alpha \) mice. (D) Tear secretion by pilocarpine adjusted for the weight of each lacrimal gland. (E) Time course of tear secretion in each 5-min period after pilocarpine administration in wild-type (diamond), \( \text{Itpr}^2/\alpha \) (triangle), \( \text{Itpr}^3/\alpha \) (cross), and \( \text{Itpr}^2/\alpha - \text{Itpr}^3/\alpha \) (square) mice. (F) The tear secretion by epinephrine adjusted for weight of each lacrimal gland. All data are presented as means ± standard error of the mean (SEM). Student’s t-test, *P < 0.05; **P < 0.01. All experiments were performed at least three times, and representative data are shown.

doi:10.1371/journal.pone.0099205.g001

**Figure 2. Lack of acetylcholine- and epinephrine-induced Ca\(^{2+}\) signals in lacrimal glands in \( \text{Itpr}^2/\alpha - \text{Itpr}^3/\alpha \) mice.** (A and B) Western blot analysis of lacrimal glands from wild-type, \( \text{Itpr}^2/\alpha \), \( \text{Itpr}^3/\alpha \), and \( \text{Itpr}^2/\alpha - \text{Itpr}^3/\alpha \) mice, using IP3R antibodies. (C) Immunohistochemistry of IP3R3 in wild-type and \( \text{Itpr}^3/\alpha \) lacrimal glands. Each panel indicates IP3R3 (green), DAPI (blue), visible image, and the merged image, respectively. Scale bar, 30 \( \mu \)m. All experiments were performed at least three times, and representative data are shown. (D) Dose-dependent Ca\(^{2+}\) response of lacrimal gland acinar cells. (E) Quantitation of Ca\(^{2+}\) peak amplitude. Lacrimal gland acinar cells were sequentially stimulated with 0.3, 1.0, and 3.0 \( \mu \)M acetylcholine. All data are presented as means ± SEM. Student’s t-test, *P < 0.05; **P < 0.01. All experiments were performed at least three times, and representative data are shown. (F) Ca\(^{2+}\) signals in response to the epinephrine (5, 10 \( \mu \)M) stimulation. Ten \( \mu \)M CPA, a SERCA pump inhibitor, was applied to check the Ca\(^{2+}\) store within the ER of \( \text{Itpr}^2/\alpha - \text{Itpr}^3/\alpha \) lacrimal acinar cells. (G) Quantitation of Ca\(^{2+}\) peak amplitude induced by 5 \( \mu \)M epinephrine.

doi:10.1371/journal.pone.0099205.g002
a polivinylidene difluoride membrane. The membrane was blocked with 5.0% skim milk in 0.05% Tween/phosphate-buffered saline (PBST) for 1 h and probed with the indicated primary antibodies. The primary antibodies KM1112, KM1083, and KM1082 were used to detect IP_{3}R1, IP_{3}R2, and IP_{3}R3, respectively [8]. The Pan-IP_{3}R antibody is an antibody that recognizes the consensus epitope of all types of IP_{3}Rs [9]. Anti-β-actin antibody (AC-15) was purchased from Sigma (Tokyo, Japan).

Incubation of the membrane with the primary antibody was performed for 2 h at room temperature. After washed with PBST, the membrane was further incubated with horseradish peroxidase-labeled secondary antibodies (1:4000; GE Healthcare, Amersham, UK) for 1 h at room temperature, and the immobilized specific antigen was visualized with the ECL plus detection kit (GE Healthcare).

**Measurement of Tear Secretion**

The mice were anesthetized by intraperitoneal injection of 36 mg/kg ketamine (Daichi Sankyo, Tokyo, Japan) and 16 mg/kg xylazine (Bayer HealthCare, Leverkusen, Germany). Tear production was stimulated by intraperitoneal injection of 3 mg/kg pilocarpine (Santen, Osaka, Japan) or 1 mg/kg epinephrine at 1 min after the anesthesia. Tears were collected for 15 min and the volume was calculated every 5 min during the 15-min duration using 0.5-μL capillary microglass tubes (Drummond, PA, USA). After the measurement, the mice were sacrificed, and the lacrimal glands were extirpated. Then, the lacrimal gland weights were measured, and the mean values were calculated to obtain the average lacrimal gland weight of the mice. The tear secretion volume was adjusted for the weight of each lacrimal gland.

**Histopathology and Electron Microscopy**

For histopathology, the extracted lacrimal glands and conjunctiva were embedded in an optimal cutting temperature compound (Sakura Finetechical, Tokyo, Japan). Frozen sections (5-μm thick) of the lacrimal glands or the conjunctiva were fixed with 10% formalin neutral buffer solution (Wako) and stained with hematoxylin and eosin or with the periodic acid-Schiff reagent. For electron microscopic observation, a portion of the lacrimal glands was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer overnight and was post-fixed with 1.0% osmic acid in 0.1 M cacodylate buffer. The specimens were dehydrated with ethanol and embedded in epoxy resin. The ultra-thin sections (80 nm) were double-stained with uranyl acetate and lead citrate, and were examined under a transmission electron microscope (1200 EXII; JEOL, Tokyo, Japan).

**Immunohistochemical Analysis**

Immunohistochemical analysis for IP_{3}R3 localization and concentration in lacrimal gland sections from wild-type, {Itpr3}^{-/-}, and {Itpr2}^{-/-}{Itpr3}^{-/-} mice. The extracted lacrimal glands were embedded in an optimal cutting temperature compound. The frozen sections (5-μm thick) were fixed with 10% formalin neutral buffer solution (Wako) and incubated with antibodies against IP_{3}R3 (1:250; BD Transduction Laboratories, Heidelberg, Germany), CD45, F4/80, CD19, CD8, or CD4 (1:100; eBioscience, San Diego, CA, USA). Signals were detected by incubating with rabbit anti-mouse IgG antibodies conjugated with Alexa 488 or peroxidase (Dako, Glostrup, Denmark). Peroxidase-conjugated antibodies were visualized by adding diaminobenzidine tetrahydroxychloride. Nuclear staining was performed with 4'-6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan) or hematoxylin.

**Measurement of Acinar Cell Area of the Lacrimal Glands**

For quantitative analysis, hematoxylin/eosin (HE)-stained sections of the lacrimal glands from wild-type and {Itpr2}^{-/-}{Itpr3}^{-/-} mice were used. The lacrimal acinar cell area was measured as reported previously [10].

**Measurement of Intracellular Ca^{2+} Concentration in Lacrimal Gland Cell Suspensions**

Following deep anesthesia by the intraperitoneal injection of 60 mg/kg nembutal (Dainippon Sumitomo Pharma, Osaka, Japan), the mice were sacrificed. Subsequently, the exorbital lacrimal glands were immediately removed, placed in cold balanced salt solution (BSS) containing 115 mM NaCl, 5.4 mM KCl, 2 mM Ca^{2+}, 1 mM Mg^{2+}, 20 mM Heps, and 10 mM glucose (pH7.4), and rapidly minced under exposure to 2 mg/mL collagenase type 2 (Worthington, Malvern, PA, USA) in BSA. The material was then digested for 10 min at 37°C with 2 mg/mL collagenase type 2 in BSS, the suspension being gently passed through a pipette several times. After the digestion, 1 mL of BSS was added to the preparation and then centrifuged at 100 kg for 3 min. The pellet was rinsed in 1 mL BSS and centrifuged in order to collect the lacrimal gland cells.

The isolated lacrimal gland cell preparation was incubated in 5 μM fura-2 AM (Dojindo)/BSS for 45 min at room temperature, rinsed twice, resuspended in 500 μL of BSS, and stored at 4°C. For the two-dimensional measurement of Ca^{2+} changes, a 75-μL sample of fura-2-loaded lacrimal gland cells was dispersed on a Cell-Tak (BD Biosciences, Bedford, MA, USA)-coated glass coverslip that formed the bottom of the recording chamber, mounted on the stage of an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan), and perfused with BSS at a rate of 2 mL/min at room temperature. Excitation of fura-2 was performed every 5 s by alternate illumination with 340 and 380 nm light. The resultant fluorescence (510–550 nm; F340/F380) was imaged using an objective lens (UPlanApo 20x/340, Olympus) and a silicon-intensified target camera to obtain pseudo-colored images of F340/F380, and stored in a personal computer using the ARGUS50/CA software (Hamamatsu Photonics, Shizuoka, Japan). The peak amplitude Ca^{2+} responses (R, delta Fura-2 ratio 340/380) were expressed as the averaged amplitude from 0–50 sec was equal to zero.

**Real Time RT-PCR**

Total RNA was extracted from cells in the lacrimal glands of the mice using the TRIZol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Complementary DNA was produced from total RNA using Superscript VILO™ Master Mix (Invitrogen). Quantitative real-time PCR was performed using the StepOnePlus Real Time PCR system (Applied Biosystems) with Fast Advanced Master Mix (Applied Biosystems) and the predesigned primers for tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [TagMan Gene Expression Assay (TNF-α: Mm00443258-m1, IL-6: Mm00446190-m1, GAPDH: Mm9999915-g1)]. The mRNA levels were evaluated by the ΔACT method, and normalized to GAPDH mRNA.

**Enzyme-linked Immunosorbent Assay (ELISA) for Immunoglobulins and Auto-antibodies**

The amounts of mouse immunoglobulins and auto-antibodies in sera from wild-type and {Itpr2}^{-/-}{Itpr3}^{-/-} mice were analyzed by ELISA. For the detection of antibodies to SS-A antigens, the
mouse sera were diluted 1:100 and analyzed using mouse anti-SS-A IgG ELISA kits (Alpha Diagnostics, San Antonio, TX, USA).

Statistical Analysis
All summarized data were expressed as means ± SEM. Statistical significance was calculated by unpaired Student’s t-test or Mann–Whitney U-test. A p value less than 5% was considered statistically significant.

Results
Itpr2<sup>-/-;</sup>Itpr3<sup>-/-</sup> Mice had Severe Defects in Tear Secretion Via Both Cholinergic and Adrenergic Receptor Pathways
We have previously reported that IP<sub>3</sub>R2 and IP<sub>3</sub>R3 play crucial roles in secretions from salivary, pancreatic, and nasal glands [6,7]. However, the subtypes of IP<sub>3</sub>R expressed in lacrimal glands and their contribution to tear secretion remain unknown. To analyze the role of IP<sub>3</sub>Rs in lacrimal glands, we measured tear flow in mice deficient in IP<sub>3</sub>Rs (Fig. 1A). Since the body weight and lacrimal gland weight were different between wild-type and mutant mice (Figs. 1B, 1C), the tear volume was normalized against lacrimal gland weight. After the intraperitoneal administration of pilocarpine, a cholinergic receptor agonist, wild-type mice shed a large volume of tears in a time-dependent manner (Fig. 1D, E). Tear secretion in Itpr2<sup>-/-;</sup>Itpr3<sup>-/-</sup> mice was comparable with that in wild-type mice, while Itpr3<sup>-/-</sup> mice shed more tears than the wild-type mice. In contrast, tear secretion was abolished in Itpr2<sup>-/-;</sup>Itpr3<sup>-/-</sup> mice (Fig. 1D).

We also examined the contributions of IP<sub>3</sub>Rs in tear secretion via the sympathetic pathway. As shown in Fig. 1F, tear flow by intraperitoneal administration of epinephrine was clearly observed in wild-type mice, but not in Itpr2<sup>-/-;</sup>Itpr3<sup>-/-</sup> mice. These results suggest that IP<sub>3</sub>R2 and IP<sub>3</sub>R3 are the predominant subtypes of

Figure 3. Altered ocular surface in Itpr2<sup>-/-;</sup>Itpr3<sup>-/-</sup> mice. (A) Anterior segment photos of the ocular surface. Wild-type and Itpr2<sup>-/-;</sup>Itpr3<sup>-/-</sup> mice corneas were viewed and photographed under white light. Debris is indicated by white arrowheads. Bar: 1 mm. (B) Histological detection of conjunctiva mucins stained with periodic acid-Schiff base. The conjunctiva of Itpr2<sup>-/-;</sup>Itpr3<sup>-/-</sup> mice had abundant mucin complexes (arrow head). Scale bar: 50 μm. (C, D) Anterior segment photos of ocular surface fluorescein staining, and the score. Bar: 1 mm. (E) Comparison of spontaneous blink rate. All data are presented as means ± SEM. Student’s t-test, *P<0.05. All experiments were performed at least three times, and representative data are shown.

doi:10.1371/journal.pone.0099205.g003
IP3Rs in lacrimal glands and are essential for tear secretion via both the cholinergic and sympathetic pathways.

Acetylcholine- and Epinephrine-induced Ca2+ Signals are Abolished in Itpr2−/−;Itpr3−/− Lacrimal Acinar Cells

We next examined the expression level of each IP3R subtype in the lacrimal glands. We found that all three types of IP3Rs were expressed in mouse lacrimal glands (Fig. 2A). No bands were detected with anti-Pan-IP3R antibodies in the Itpr2−/−;Itpr3−/− lacrimal gland lysates (Fig. 2A). In addition, IP3Rs were detected by anti-Pan-IP3R antibodies in lacrimal gland lysates from Itpr2−/− but not in Itpr3−/− mice (Fig. 2B), suggesting that IP3R3 exhibits the highest expression level among the three subtypes. Immuno-histochemical studies using the anti-IP3R3 antibody revealed that IP3R3 is localized at the restricted region near the apical membranes in the acinar cells where endocrine secretion occurs (Fig. 2C). IP3R3 fluorescein staining was not detectable in Itpr3−/− mice (Fig. 2C).

Ca2+ transients were clearly observed in response to acetylcholine (Ach) in wild-type lacrimal gland acinar cells in a dose-dependent manner (Fig. 2D). The Itpr2−/− and Itpr3−/− acinar cells showed Ca2+ responses that were comparable to those of the wild-type cells, except that the Itpr3−/− cells exhibited relatively rather long-lasting Ca2+ signals with decreased peak amplitudes, especially at 3.0 μM Ach (Figs. 2D, 2E). These long-lasting Ca2+ signals were likely due to the nature of the residual IP3R2, which has the highest affinity for IP3 among the three types of IP3Rs, and might explain the larger amount of tear secretion in Itpr3−/− mice (Fig. 1D). In contrast, Ach-induced Ca2+ transients were diminished in the Itpr2−/−;Itpr3−/− acinar cells (Figs. 2D, 2E).

Moreover, Itpr2−/−;Itpr3−/− acinar cells exhibited no epinephrine-induced Ca2+ transients (Fig. 2F, G). The diminished Ca2+ signals in the Itpr2−/−;Itpr3−/− acinar cells on epinephrine stimulation was not due to the depletion of Ca2+ stores, because cyclopiazonic acid (CPA), a Ca2+ pump inhibitor, induced a considerable Ca2+ leak from the endoplasmic reticulum (Fig. 2F). These results suggest that IP3R2 and IP3R3 are essential for Ca2+ signals in both the sympathetic and parasympathetic pathways.

Figure 4. Histological analysis of lacrimal gland tissues. (A) Tissue sections of lacrimal glands from wild-type and Itpr2−/−;Itpr3−/− mice were stained by hematoxylin/eosin (HE) and observed under light microscopy. White arrowheads indicate inflammatory infiltrates. Scale bar: 50 μm. (B) Electron micrographs of lacrimal glands from wild-type and Itpr2−/−;Itpr3−/− mice. Scale bar: upper panels, 5 μm; lower panels, 2 μm. All experiments were performed at least three times, and representative data are shown. N; Nucleus, lu; lumen, ER; endoplasmic reticulum. (C) Relative lacrimal acinar cell area. The acinar cell area of wild-type (n = 54) and Itpr2−/−;Itpr3−/− (n = 59) lacrimal acinar cells was measured using HE-stained sections. Values represent the means ± SEM. Student’s t-test, ***, P < 0.001.

doi:10.1371/journal.pone.0099205.g004
Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup> Mice cause Dry Eye

We carefully checked the ocular surfaces of Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup> mice. A significant amount of debris was observed on the corneal surfaces in Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup> mice (Fig. 3A). Abnormalities of the conjunctival surface bound to abundant mucin complex were observed in Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup> mice (Fig. 3B). A reduction in the number of goblet cells, a common feature of dry eye patients, was also observed in Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup> mice. In addition, Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup> mice showed increased corneal fluorescein staining at 6 weeks (Figs. 3C, D), which indicates corneal epithelial barrier disruption in these mutant mice. This was not due to the abnormal development of the corneal surface, because no significant difference was observed in corneal staining between the ocular surfaces of wild-type and Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup> mice at 3 weeks after birth, immediately after the mice opened their eyes (data not shown). Moreover, Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup> mice showed increased blink rates because of insufficient tear flow on the ocular surface (Fig. 3E).

Figure 5. Infiltration of inflammatory mononuclear cells in Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup> lacrimal glands. (A) Immunostaining of CD45, F4/80, CD19, CD8 and CD4 in lacrimal gland tissue sections from wild-type and Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup> mice. White arrowheads indicate inflammatory mononuclear cells. (B) Quantification of TNF-α mRNA expression levels by real time RT-PCR. Six week-old mice; wild-type: n = 8 and Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup>: n = 8. Ten week-old mice; wild-type: n = 16, Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup>: n = 10. Mann-Whitney U-test, ***P<0.001, *P<0.05. All data are presented as means ± SEM. (C) Quantification of IL-6 mRNA expression levels by real time RT-PCR. Six week-old mice; wild-type: n = 8 and Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup>: n = 8. Ten week-old mice; wild-type: n = 16, Itpr2<sup>−/−</sup>;Itpr3<sup>−/−</sup>: n = 10. Mann–Whitney U-test, ***P<0.001. All data are presented as means ± SEM.

doii:10.1371/journal.pone.0099205.g005
Dry Eye Mice with Altered Lacrimal Ca\textsuperscript{2+} Signals

A

![Bar charts showing IgG, IgA, IgG1, IgG2a, and IgG3 levels with wild-type and Ltpr2\textsuperscript{-/-};Ltpr3\textsuperscript{-/-} comparisons.](chart)

B

![Line graph showing Anti-SS-A (µg/ml) levels with week comparisons](chart)
Atrophy of the Lacrimal Glands in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} Mice

We next performed histological analysis of the lacrimal gland tissue, and found atrophy of the lacrimal gland acinar units with marked lymphocytic infiltration in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice more than 10 weeks of age (Fig. 4A). Electron micrographs also demonstrated the distinct morphology of acinar cells between wild-type and \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice. Secretory vesicles were located near the acinar lumen side and the well-developed endoplasmic reticulum (ER) structure was clearly observed in the cytoplasm near the apical side of the wild-type lacrimal acinar cells (Fig. 4B). In the \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} acinar cells, however, an excessive number of secretory vesicles accumulated and distributed in the cytoplasm, making it difficult to detect the ER in the cytoplasm (Fig. 4B). We also found that the \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} acinar cells seemed to be smaller than wild-type acinar cells. The lacrimal acinar cell area in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice was approximately 40% smaller than that in wild-type mice (Fig. 4C).

Inflammation of the Lacrimal Glands in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} Mice

To further explore the inflammation state of the lacrimal glands in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice, we classified the inflammatory infiltrates by using several lymphocyte markers (leukocyte; CD45, macrophage; F4/80, T-cell; CD4 and CD8, B-cell; CD19). We found that CD45-positive inflammatory mononuclear cells infiltrated the lacrimal glands in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice at 10 weeks (Fig. 5A, left panel, white arrow heads). These CD45-positive cells were located in the interstitial space around the lacrimal gland acinar cells. Macrophages and activated T-cells were the major inflammatory cells at 10 weeks (Fig. 5A); however, the population of infiltrating cells changed thereafter, and many B cells were detected at 40 weeks (Fig. 5A, right panel, arrow). We also checked the inflammatory environment of the lacrimal glands by evaluating the levels of pro-inflammatory cytokines. We found that the expression levels of pro-inflammatory cytokines such as TNF-\textgamma and IL-6 were significantly increased in the lacrimal glands in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice (Fig. 5B and C).

\textit{Itp2}^{-/-};\textit{Itp3}^{-/-} Mice Present Autoantibodies against Ribonucleoprotein SSA

We finally examined the concentrations of immunoglobulins and autoantibodies against ribonucleoprotein SSA, one of the most commonly detected autoantibodies in patients with SS, in the serum of \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice. As shown in Fig. 6A, we found that the concentration of immunoglobulin was significantly higher in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice than in wild-type mice. Moreover, the levels of autoantibodies against SSA were significantly higher in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice compared to wild-type mice at 10 weeks, when the infiltrates were observed (Fig. 6B).

\section*{Discussion}

In this study, we have shown that the type 2 and type 3 IP\textsubscript{3}Rs are predominantly expressed in lacrimal glands and that IP\textsubscript{3}Rs are essential for tear secretion via both the sympathetic and parasympathetic signaling pathways. We also found that Ca\textsuperscript{2+} signals in response to epinephrine as well as cholinergic receptors were diminished in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} lacrimal gland cells. The lack of tear flow resulted in increased eye blink rates, and the corneal surface and conjunctiva were severely damaged in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice. As the mutant mice aged, \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice displayed atrophy and infiltration of lacrimal glands as well as the production of autoantibodies against SSA in the sera, which are clinical features observed in human SS [11,12]. Thus, our \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice constitute a novel dry eye mouse model with an SS-like phenotype.

It is well known that norepinephrine released from sympathetic nerves predominantly activates \textalpha;-adrenergic receptors and induces Ca\textsuperscript{2+} elevation in lacrimal acinar cells [13]. However, in contrast to the established role of IP\textsubscript{3}R in Ca\textsuperscript{2+} elevation induced by parasympathetic stimuli, the Ca\textsuperscript{2+} channels that are responsible for cytosolic Ca\textsuperscript{2+} elevation triggered by \textgamma;-adrenergic stimuli are not clearly identified in lacrimal acinar cells. Several previous studies suggested a role for ryanodine receptors in Ca\textsuperscript{2+} elevation in lacrimal glands by norepinephrine [3]. Our study clearly demonstrated that IP\textsubscript{3}Rs contribute significantly to adrenergic tear secretion as well as cholinergic tear secretion in vivo. Ca\textsuperscript{2+} transients triggered by epinephrine were diminished in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} lacrimal gland acinar cells. These results suggest that Ca\textsuperscript{2+} release from IP\textsubscript{3}Rs is a crucial event in both cholinergic and adrenergic signal transduction in lacrimal glands, which underlies the lack of tear secretion, resulting in the abnormal ocular surface seen in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice.

It is an important observation that \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice developed only corneal and conjunctival injuries at 6 weeks of age and showed lacrimal gland infiltrations only after 10 weeks of age. Thus, ocular surface disturbance seems to occur prior to lymphocyte infiltration into the lacrimal glands in \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice. Together with the previous finding that the desiccating stress of the ocular surface induces lacrimal gland inflammation and infiltration [14], corneal surface and conjunctival injuries caused by long-lasting dysfunction of lacrimal acinar cells may lead to the activation of antigen-presenting cells [15] and the subsequent breakdown of self-tolerance against endogenous epitopes shared among lacrimal gland units. Further studies are necessary for a clear understanding of the mechanism of infiltration in the lacrimal glands, which might contribute to the pathogenesis of SS in humans.

In conclusion, we have demonstrated that IP\textsubscript{3}R2 and IP\textsubscript{3}R3 play a central role in tear secretion and maintenance of the lacrimal glands. Our data indicate that Ca\textsuperscript{2+} release from IP\textsubscript{3}Rs in lacrimal gland acinar cells is essential for sympathetic as well as cholinergic tear secretion. Together with the defect in salivary secretion observed in our previous study [6], the diversified symptoms of \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice including lacrimal gland inflammatory foci, ocular surface disruption, and the production of autoantibodies against SSA fulfill the criteria for a diagnosis of SS, established by the American-European Consensus Group [16]. We believe that \textit{Itp2}^{-/-};\textit{Itp3}^{-/-} mice will be a useful tool for the analysis of pathological mechanisms and for the development of new treatment strategies for SS.
Acknowledgments

We would like to thank Dr. Toshihiro Nagai, Dr. Katsuhiro Kawaai, and Dr. Takeyuki Sugawara for their excellent technical advice, Ms Kotomi Sugiura for mouse breeding, and Dr. Murat Dogru and Dr. Tomoko Sato for their critical reading of the manuscript. We also thank Dr. Ichiro Saito, Dr. Kensei Tsuzaka, Dr. Akihiro Mizutani, Dr. Yukiko Kuroda, and Dr. Helen Kiefer for valuable discussion.

Author Contributions

Conceived and designed the experiments: KM CH TI. Performed the experiments: CH TI YS YO EE NO. Analyzed the data: CH TI TT. Contributed reagents/materials/analysis tools: MM. Wrote the paper: CH TI KM KT.

References

1. Dartt DA (1989) Signal transduction and control of lacrimal gland protein secretion: a review. Curr Eye Res 8: 619–636.
2. Dartt DA (1994) Regulation of tear secretion. Adv Exp Med Biol 350: 1 – 9.
3. Gromada J, Jorgensen TD, Dissing S (1995) The release of intracellular Ca²⁺ in lacrimal acinar cells by α-, β-adrenergic and muscarinic cholinergic stimulation: the roles of inositol triphosphate and cyclic ADP-ribose. Pflugers Arch 429: 751–761.
4. Hodges RR, Dicker DM, Rose PE, Dartt DA (1992) α-1-adrenergic and cholinergic agonists use separate signal transduction pathways in lacrimal gland. Am J Physiol 262: G1087–1096.
5. Dartt DA (2009) Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. Prog Retin Eye Res 28: 155–177.
6. Futatsugi A, Nakamura T, Yamada MK, Ebisui E, Nakamura K, et al. (2005) IP₃ receptor types 2 and 3 mediate exocrine secretion underlying energy metabolism. Science 309: 2232–2234.
7. Fukuda N, Shirasu M, Sato K, Ebisui E, Touhara K, et al. (2008) Decreased olfactory mucus secretion and nasal abnormality in mice lacking type 2 and type 3 IP₃ receptors. Eur J Neurosci 27: 2665–2675.
8. Iwai M, Tateshi Y, Hattori M, Minatani A, Nakamura T, et al. (2005) Molecular cloning of mouse type 2 and type 3 inositol 1,4,5-trisphosphate receptors and identification of a novel type 2 receptor splice variant. J Biol Chem 280: 10365–10377.
9. Hattori M, Suzuki AZ, Higo T, Miyauchi H, Michikawa T, et al. (2004) Distinct roles of inositol 1,4,5-trisphosphate receptor types 1 and 3 in Ca²⁺ signaling. J Biol Chem 279: 11967–11975.
10. Kamoi M, Ogawa Y, Nakamura S, Dogru M, Nagai T, et al. (2012) Accumulation of secretory vesicles in the lacrimal gland epithelia is related to non-Sjogren’s type dry eye in visual display terminal users. PLoS One 7: e43638.
11. Fox RI (1995) Sjogren’s syndrome. Curr Opin Rheumatol 7: 409–416.
12. Fox RI, Maruyama T (1997) Pathogenesis and treatment of Sjogren’s syndrome. Curr Opin Rheumatol 9: 393–399.
13. Dartt DA, Rose PE, Dicker DM, Ronco LV, Hodges RR (1994) Alpha 1-adrenergic agonist-stimulated protein secretion in rat exorbital lacrimal gland acini. Exp Eye Res 58: 423–429.
14. Niederkorn JY, Stern ME, Plungfelder SC, De Paiva CS, Corrales RM, et al. (2006) Desiccating stress induces T cell-mediated Sjogren’s Syndrome-like lacrimal keratoconjunctivitis. J Immunol 176: 3950–3957.
15. Matzinger P (2002) The danger model: a renewed sense of self. Science 296: 301–305.
16. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, et al. (2002) Classification criteria for Sjogren’s syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 61: 534–538.