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Role of *Orai1* and store-operated calcium entry in mouse lacrimal gland signalling and function

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Key points

- Lacrimal acinar cells from mice whose gene for Orai1 has been deleted have no detectable store-operated Ca^{2+} entry, whether assessed by measurement of cytoplasmic Ca^{2+} changes or as a store-operated current.
- Mice lacking Orai1 have diminished lacrimal fluid secretion in response to muscarinic–cholinergic stimulation.
- Mice lacking Orai1 also show diminished exocytosis, both *in vivo* and *in vitro*.
- The development and morphology of lacrimal glands, as well as responses not dependent on Ca^{2+} entry were unchanged in the knockout mice.
- The results demonstrate the central importance of store-operated Ca^{2+} entry in lacrimal exocrine function, and suggest possible strategies for combating diseases associated with diminished lacrimal secretion.

Abstract Lacrimal glands function to produce an aqueous layer, or tear film, that helps to nourish and protect the ocular surface. Lacrimal glands secrete proteins, electrolytes and water, and loss of gland function can result in tear film disorders such as dry eye syndrome, a widely encountered and debilitating disease in ageing populations. To combat these disorders, understanding the underlying molecular signalling processes that control lacrimal gland function will give insight into corrective therapeutic approaches. Previously, in single lacrimal cells isolated from lacrimal glands, we demonstrated that muscarinic receptor activation stimulates a phospholipase C-coupled signalling cascade involving the inositol trisphosphate-dependent mobilization of intracellular calcium and the subsequent activation of store-operated calcium entry (SOCE). Since intracellular calcium stores are finite and readily exhausted, the SOCE pathway is a critical process for sustaining and maintaining receptor-activated signalling. Recent studies have identified the Orai family proteins as critical components of the SOCE channel activity in a wide variety of cell types. In this study we characterize the role of Orai1 in the function of lacrimal glands using a mouse model in which the gene for the calcium entry channel protein, Orai1, has been deleted. Our data demonstrate that lacrimal acinar cells lacking Orai1 do not exhibit SOCE following activation of the muscarinic receptor. In comparison with wild-type and heterozygous littermates, Orai1 knockout mice showed a significant reduction in the stimulated tear production following injection of pilocarpine, a muscarinic receptor agonist. In addition, calcium-dependent, but not calcium-independent exocytotic secretion of peroxidase was eliminated in glands from knockout mice. These studies indicate a critical role for Orai1-mediated SOCE in lacrimal gland signalling and function.

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Abbreviations CRAC, calcium release-activated calcium; DVF, divalent cation free; HBSS, Hepes-buffered saline solution; IP_3, inositol 1,4,5-trisphosphate; KO, knockout; MeCh, methacholine; Orai1-KO, Orai1 knockout; SOCE, store-operated Ca^{2+} entry; WT, wild type.

Introduction

The exocrine lacrimal glands maintain and protect the integrity of the ocular surface by providing a tear film composed of water, electrolytes, surfactants and proteins (Dartt, 2009). Generation and composition of the tear film are under tight neural control so that it can respond rapidly to environmental changes that may occur at the ocular surface. Dysfunction of lacrimal secretion underlies a number of debilitating diseases of the eye (Dartt, 2004). Lacrimal glands are responsive to both muscarinic and α-adrenergic stimulation, and the secretion of both fluid and protein appear to be dependent on the phospholipase C-coupled calcium signalling system (Dartt, 1989; Putney & Bird, 1998).

In addition to their importance to ocular health, the study of signalling pathways in lacrimal cells has been instrumental in defining critical aspects of the underlying phospholipase C-coupled calcium signalling cascade. Activation of muscarinic cholinergic receptors leads to robust formation of inositol 1,4,5-trisphosphate (IP_3) (Godfrey & Putney, 1984) and sustained elevations in cytoplasmic Ca^{2+} (Kwan *et al.* 1990). Lower, more physiological levels of activation induce cytoplasmic Ca^{2+} oscillations (Bird *et al.* 1993). Receptor activation can be bypassed either by uncaging of an agonist for IP_3 receptors (Bird *et al.* 1992*b*), by use of Ca^{2+} ionophores (Bird *et al.* 1992*a*) or by inhibition of sarcoplasmic–endoplasmic reticulum calcium ATPase pumps with thapsigargin (Kwan *et al.* 1990; Bird *et al.* 1992*a*). In the last case, depletion of endoplasmic reticulum Ca^{2+} stores by thapsigargin leads to the activation of plasma membrane store-operated channels. Indeed, experiments with lacrimal cells published over 30 years ago (Parod & Putney, 1978) revealed for the first time that the refilling of intracellular Ca^{2+} stores occurred independently of receptor activation, which ultimately led to the concept of store-operated calcium entry (SOCE; Putney, 1986).

We now understand that SOCE most commonly involves highly Ca^{2+}-selective calcium release-activated calcium (CRAC) channels (Hoth & Penner, 1992). The signal for activation of these channels comes from endoplasmic reticulum-resident STIM proteins that activate one or more of the family of Orai (1–3) channel proteins in the plasma membrane (Cahalan *et al.* 2007; Hogan *et al.* 2010). However, it is not known whether SOCE in lacrimal cells involves CRAC channels, or whether the channels consist of Orai subunits. And despite the well-documented role of store-operated entry in Ca^{2+} signalling in lacrimal cells *in vitro*, the role of this pathway in *in vivo* secretion is not known.

In recent years, the development of mouse models deficient in these proteins has been key to understanding the regulation of SOCE, and its physiological context in the intact animal. In this study, we employ a mouse model deficient in the Orai1 channel protein (Orai1-knockout (KO), generated by gene trap (Vig *et al.* 2008)) to investigate the role of Orai1 in lacrimal gland function and calcium signalling. We find that sustained calcium signalling, as well as activated lacrimal secretion, is substantially compromised in the absence of Orai1.

Methods

Ethical approval and animal procedures

All animal procedures were reviewed and approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee (NIEHS ACUC). All animals were housed, cared for and used in compliance with the Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf).

When mice were euthanized for tissue collection, they were placed in a clear, air-filled chamber (10 litre) and then exposed to CO_2 at a rate of 2 l min^{-1}. After the animals became unconscious and stopped breathing, the CO_2 flow was continued for at least 5 min to avoid recovery when removed from the chamber. A thoracotomy was performed before tissue collection commenced.

For experiments involving measurement of fluid secretion in anaesthetized mice, at the end of the experiment some mice were euthanized for collection of tissue for histological and electron microscopic analysis, and others were allowed to recover. Following anaesthesia, mice were kept on a warming pad and monitored for any adverse effects until fully recovered.

The total number of mice used in this study was 77 (wild-type (WT) 38; KO 39).

Orai1-KO mice

Orai1-KO mice (C57/DBA/129 background; Vig *et al.* 2008) generally died perinatally, but surviving pups

(male and female) were obtained from mice crossed with out-bred ICR mice (Harlan Laboratories Inc. (Indianapolis, IN, USA) strain Hsd: ICR (CD-1)) for two generations. Litters of more than six pups were split and fostered by Swiss Webster mice. Litters with potential KO pups, as suggested by small pup size (Vig *et al.* 2008), were kept on heating pads until weaning. Mice were typically weaned and genotyped by tail biopsy at 21 days. Potential KO mice were kept with the mother/foster mouse until they were able to eat on their own, usually ∼4 weeks. Due to the infrequency of obtaining surviving KO mice, mice were used at varying ages, but always paired to WT littermates. The average age of mice for the study was 17 weeks.

Isolation of mouse lacrimal acinar cells

Mice were killed and exorbital lacrimal glands excised, finely minced and subjected to enzymatic digestion as previously described (Bird *et al.* 1992*a*). Isolated cells were washed and suspended in DMEM containing 10% fetal bovine serum, 5 mM glutamine, 100 units ml^{-1} penicillin and 100 units ml^{-1} streptomycin. Cells were allowed to attach to glass coverslips (no. 1.5) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

Real time RT-PCR

Total RNA was isolated and purified using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) from lacrimal acinar cells prepared from WT and Orai1-KO mice. Reverse transcription of RNA was performed using the Omniscript RT Kit (Qiagen), and resulting cDNA was amplified using TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA, USA) (Davis *et al.* 2013). The following mouse TaqMan Gene Expression Assays were used in this study: Orai1 (Mm00774349_m1), Orai2 (Mm04214089_s1), Orai3 (Mm01612888_m1), Stim1 (Mm01158413_m1), Stim2 (Mm01223103_m1) and Trpc1 (Mm00441975_m1). Reactions were cycled using an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies) with universal cycling conditions. Relative quantification was determined with reference to 18S rRNA and was analysed using the comparative CT method (Suchanek *et al.* 2002).

Single-cell calcium measurement

Lacrimal cells, plated on 30 mm round coverslips and mounted in a Teflon chamber, were incubated in DMEM with 0.5 μM fura-5F/AM (Genolite Biotek, Portland, OR, USA) at 37°C for 10 min. For $[Ca^{2+}]_i$ measurements, cells were bathed in Hepes-buffered salt solution (HBSS) at room temperature (21°C) containing (mM): NaCl 120, KCl 5.4, $MgCl_2$ 0.8, Hepes 20, $CaCl_2$ 1.8 and glucose 10; pH was adjusted to 7.4 with NaOH. Nominally Ca^{2+}-free solutions were HBSS with no added $CaCl_2$. Fluorescence images of the cells were recorded and analysed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging Inc., Cincinnati, OH, USA). Changes in intracellular calcium are expressed as the ratio of fura-5F fluorescence (F340/F380). Ratio values were corrected for contributions by autofluorescence. R_{max}, determined *in*

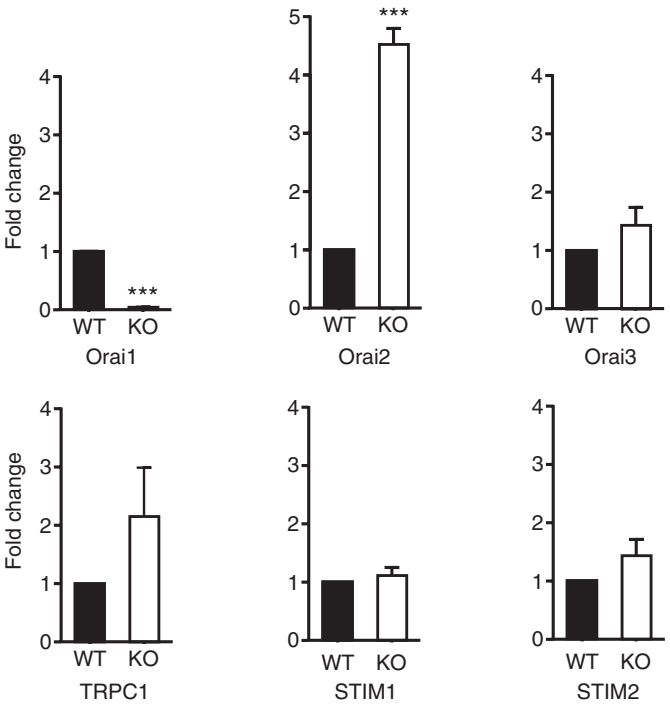

Figure 1. Gene expression levels of calcium signalling proteins in lacrimal cells isolated from wild-type (WT) and Orai1-KO (KO) mice
mRNA levels of Orai1, Orai2, Orai3, Stim1, Stim2 and TRPC1 were assessed by real-time RT-PCR as described in Methods. The data are mean ± SEM of three independent experiments (*t* test, $^{***}P < 0.001$).

situ, averaged 6.5. Peak ratio values for methacholine (MeCh) and thapsigargin were less than 3.5, indicating that the ratio values were less than saturation and thus essentially proportional to Ca^{2+} concentration.

Measurement of store-operated (I_{CRAC}) and calcium-activated potassium (I_K) currents

Lacrimal cells were attached to glass coverslips (no. 1.5) and placed on the stage of a Zeiss Axiovert 25 microscope equipped with a 40× (0.9 N.A) objective. Membrane currents were recorded at room temperature in the whole-cell configuration using an Axopatch 200B amplifier and Digidata 1322A data acquisition system, with Voltage clamp protocols and data acquisition controlled by pClamp 10 (Axon Instruments, Union City, CA, USA). Pipettes were pulled from 7740 thin-walled borosilicate capillary tubing (Sutter Instrument Co., Novato, CA, USA) and were fire polished to a tip diameter of 2∼3 μm with a resistance in bath

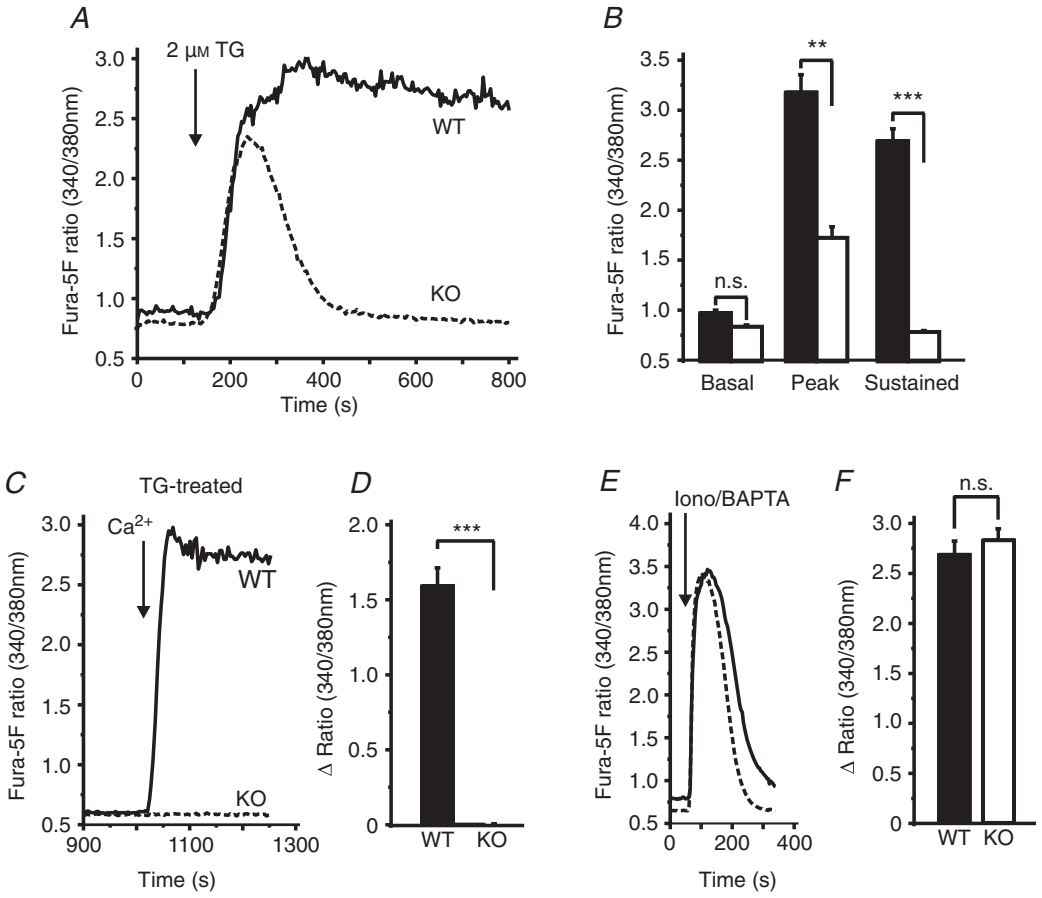

Figure 2. Thapsigargin-induced Ca^{2+} entry in lacrimal acinar cells from wild-type (WT) and Orai1 KO (KO) mice

Isolated lacrimal acinar cells were loaded with the fluorescent calcium indicator, fura-5F. *A*, responses to 2 μM thapsigargin (TG) in the presence of 1.8 mM extracellular Ca^{2+} in cells from WT (continuous line) and Orai1-KO (broken line) mice. The key characteristics of the data in *A* are summarized in *B*, including basal Ca^{2+} ratio values, peak Ca^{2+} ratio responses to thapsigargin and sustained Ca^{2+} ratio values measured at 800 s. Data are mean ± SEM for 14 cells (WT) and 18 cells (KO) captured in five independent experiments (ANOVA; $^{***}P < 0.0001$; $^{**}P < 0.05$; n.s., not significant). *C*, lacrimal cells were bathed in HBSS nominally free of extracellular Ca^{2+} and treated with 2 μM thapsigargin for 15 min, then 1.8 mM extracellular Ca^{2+} was restored to the bathing medium at 900 s. The effect of extracellular Ca^{2+} was measured as the difference in Ca^{2+} ratio values (Δ Ratio) measured before (at 850 s) and after (1200 s) the addition at 900 s. The data for WT (continuous line) and Orai1-KO (broken line) cells are summarized in *D*, as means ± SEM for 17 cells (WT) and 16 cells (KO) captured in three independent experiments (*t* test; $^{***}P < 0.0001$). *E*, response of lacrimal cells to the addition of 10 μM ionomycin in the presence of 3 mM BAPTA to assess the content of mobilizable intracellular Ca^{2+} stores in WT (continuous line) and Orai1-KO (broken line) cells, as reflected in the peak response to ionomycin/BAPTA. *F*, the effect of the extracellular Ca^{2+} was measured as the difference in Ca^{2+} ratio values (Δ Ratio) measured before (at 50 s) and after (peak response) the addition of ionomycin/BAPTA. The data are mean ± SEM for 20 cells (WT) and 19 cells (KO) captured in three independent experiments (*t* test; n.s., not significant).

solution of 2–4 MΩ. Seal resistances of 2–10 GΩ typically were obtained. Membrane potentials were corrected for the measured liquid junction potential.

For measurement of store-operated currents (I_{CRAC}), lacrimal cells were bathed in HBSS containing (in mM): 140 NaCl, 3 KCl, 10 $CsCl_2$, 1.2 $MgCl_2$, 10 $CaCl_2$, 10 glucose, and 10 Hepes (pH adjusted to 7.4 with NaOH). Divalent cation free (DVF) solution was made by removing Ca^{2+} and Mg^{2+} from the general external solution containing (in mM): 140 NaCl, 3 KCl, 10 CsCl, 10 Hepes, 10 glucose, and 0.5 EDTA. Fire-polished glass pipettes were filled with pipette solution containing (in mM): 145 cesium methanesulfonate, 10 BAPTA, 10 Hepes, 8 $MgCl_2$ and 25 μM IP_3 (pH adjusted to 7.2 with CsOH). Voltage ramps (−120 to +120 mV) of 250 ms from a holding potential of 0 mV were recorded every 2 s immediately after gaining access to the cell, and the currents were normalized based on cell capacitance.

For measurement of calcium-activated K currents (I_K), lacrimal cells were bathed in an external solution containing (in mM): 140 NaCl, 5 KCl, 1.2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 10 glucose (pH adjusted to 7.4 with NaOH). The internal pipette solution contained (in mM): 140 KCl, 2 $MgCl_2$, 2 MgATP, 10 Hepes (pH adjusted to 7.4 with KOH). IP_3, at a concentration of 10 μM, was included in the pipette solution to maximally mobilize calcium from intracellular calcium stores. The calcium buffering capacity of the internal pipette solution was adjusted with Na-BAPTA. Using 0.5 mM Na-BAPTA/0 Ca^{2+} provides a weak calcium buffer, allowing IP_3 to mobilize calcium

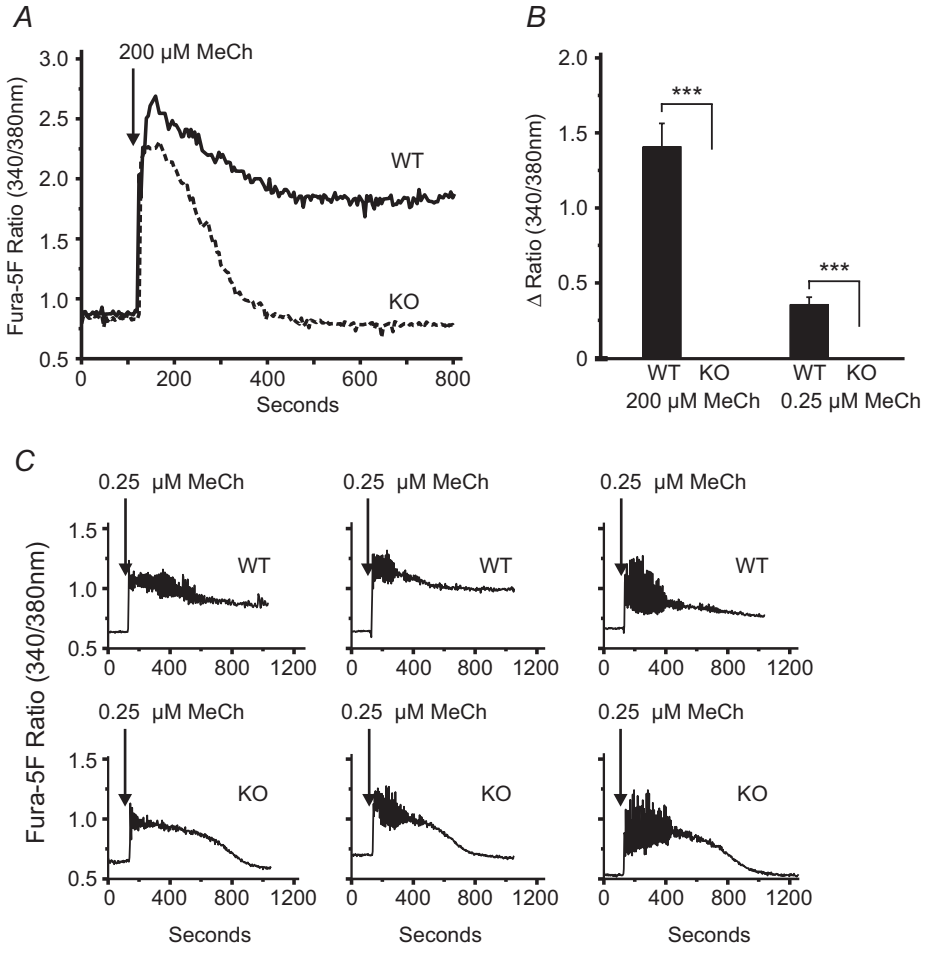

Figure 3. Methacholine-induced Ca^{2+} entry in lacrimal acinar cells from wild-type (WT) and Orai1 KO (KO) mice

Isolated lacrimal acinar cells were loaded with the fluorescent calcium indicator fura-5F. In the presence of 1.8 mM extracellular Ca^{2+}, the responses to the phospholipase C-coupled agonist MeCh were compared in cells from WT and Orai1-KO mice. *A* and *C*, responses to maximal (200 μM) and sub-maximal (0.25 μM) concentrations MeCh, respectively. *B*, summary of the sustained calcium signal above baseline (Δ Ratio), measured at 800 s for each MeCh concentration tested. For 200 μM MeCh the data are mean ± SEM for 11 cells (WT) and 14 cells (KO) and, for 0.25 μM MeCh, are mean ± SEM for 33 cells (WT) and 47 cells (KO), and captured in three independent experiments (*t* test; $^{***}P < 0.0001$).

ions to a high level in the cytoplasm ('High $Ca^{2+}{}_i$' condition) (Trautmann & Marty, 1984). In contrast, 10 mM Na-BAPTA/0 Ca^{2+} provides a strong calcium buffer, maintaining calcium ions at a low level in the presence of IP_3 ('Low $Ca^{2+}{}_i$' condition). Cells were dialysed with pipette solution for ~1 min prior to the start of recording. Successive 100 ms voltage steps were implemented from a holding potential of −40 mV to test potentials ranging from −100 to +60 mV in +10 mV increments.

Aqueous tear production: phenol red thread

WT and Orai1-KO littermates were anaesthetized (2.5% isoflurane) and then injected I.P. with pilocarpine hydrochloride (2.5 μg g^{-1} in saline). Three, 8, 15 and 21 min after mouse injection ($t = 0$), a cotton thread impregnated with phenol red was applied to the ocular surface in the lateral canthus for 60 s (Dursun *et al.* 2002). Tear production was indicated by measuring (in mm) the 'wetting' of the cotton thread. The rate of tear production was normalized against individual mouse body weight.

Histology and electron microscopy

Excised lacrimal glands were harvested from WT and KO mice, both unstimulated and stimulated with pilocarpine, and sectioned into smaller pieces (<1 μm cubes), fixed in modified Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4). The samples were bisected with one half processed for electron microscopic studies (see below) and the other half for histological examination.

For histology, the samples were dehydrated through graded alcohol, 80% × 2 for 30 min each, 95% × 2 for 45 min each, 100% × 3 for 45 min each followed by xylene × 3 30 min each. Samples were then infiltrated with four changes of paraffin, two at 30 min each and

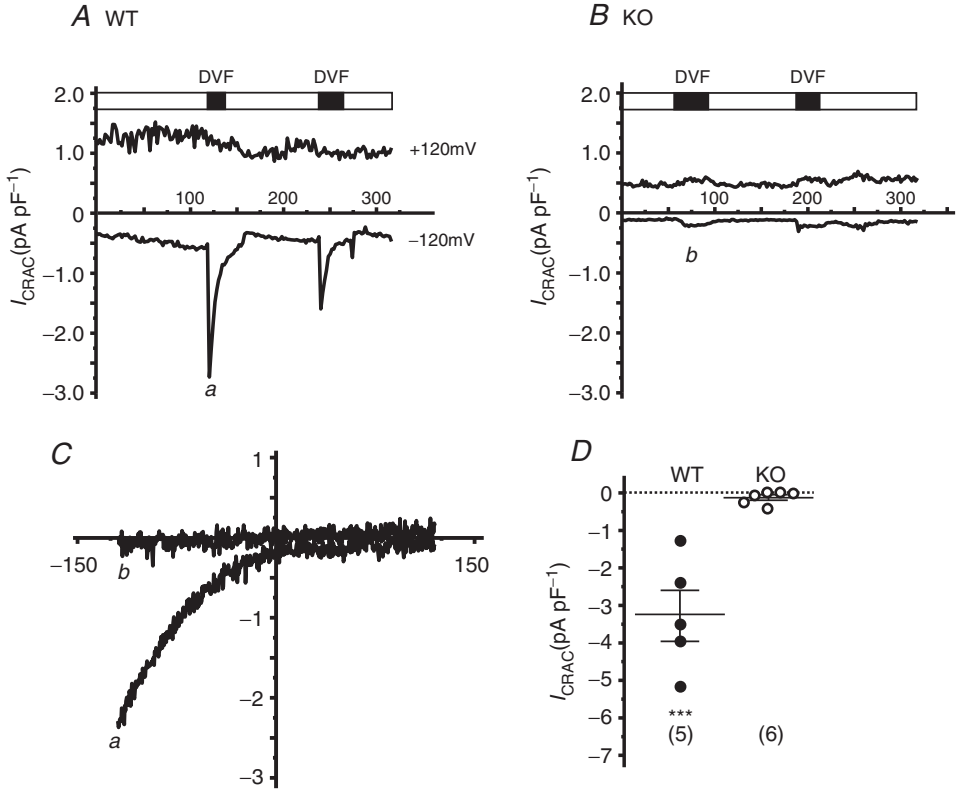

Figure 4. Store-operated currents in lacrimal acinar cells
A, patch-clamp recording of whole-cell currents in WT mouse lacrimal cells. Cells were bathed in HBSS containing 10 mM $CaCl_2$ and, on establishing a seal with the patch pipette, intracellular stores were depleted by inclusion of 25 μM IP_3 and 10 mM BAPTA in the patch pipette. Under these conditions Ca^{2+} currents clearly identifiable as I_{CRAC} could not be reliably detected. However, by switching the bathing solution to one free of all extracellular divalent ions (DVF), large transient inwardly rectifying currents appeared, a hallmark of I_{CRAC}. *B*, little or no inward current appeared in store-depleted Orai-KO cells under whole-cell or DVF conditions. *C*, leak-subtracted *I–V* current relationships measured in WT (*a* from the experiment in *A*) and Orai1-KO (*b* from the experiment in *B*) cells. *D*, summary of experiments showing large peak inward DVF currents (leak-subtracted) in WT ($n = 5$ cells) compared to little or no DVF current in Orai1-KO acinar cells ($n = 6$ cells). Horizontal bars in *D* represent the mean ± SEM (*t* test; $^{***}P < 0.005$).

two at 45 min each. The samples were then embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin (H&E). The tissue sections were placed on glass slides, mounted in Permount^{TM}, and examined with an Olympus BX51 light microscope.

For transmission electron microscopy, tissue samples were processed on a Leica EM TP automatic tissue processor. Samples were rinsed in 0.1 M sodium cacodylate buffer (four changes, 10 min each), post-fixed in 2% osmium tetroxide for 1 h, and dehydrated through graded alcohol and propylene oxide (2×, 15 min each), propylene oxide/Poly Bed (1×, 1 h), infiltrated with Poly Bed (2×, 2 h each) and then embedded in Beem capsules with Poly Bed (capsules cured at 60°C for 72 h). The blocks were then trimmed, thick sectioned (700 nm) and stained with 1% toludine blue for examination by light microscopy. Blocks were then selected to be thin sectioned (a section ~70–90 nm or 'gold'), placed on Formvar-coated copper grids then stained with uranyl acetate and lead citrate. The grids were examined on an FEI Tecnai 110KV transmission electron microscope.

Digital photomicrographs were taken of each grid/animal and examined for ultrastructural landmarks of lacrimal acinar cells. A blind morphometric analysis was performed on electron micrographs of lacrimal gland tissue prepared from WT or Orai1-KO mice that were either untreated (control) or treated (stimulated) with pilocarpine. In total, 20 electron micrographs were analysed from each mouse group: six WT-control, six KO-control, four WT-stimulated and four KO-stimulated. Identified lumens were assigned a score based on three criteria that represented the extent to which secretion had occurred. The three criteria used were: lumen dilatation, presence of membrane fragments in the lumen and empty secretory granules. A score of 1–3 was assigned based on the extent to which each of these three criteria was met. A score of 0 was assigned when a lumen was present but with none of the criteria for secretion. Based on the extent with which these criteria were met and in combination, the scores were weighted and used to calculate a 'secretion index' for each animal preparation. Data were analysed by analysis of variance (ANOVA) using PROC GLM in SAS 9.0, and with *post hoc* analysis performed using LSMEANS in PROC GLM using a standard *t* test.

Peroxidase secretion measurement

A pair of lacrimal glands was removed from each mouse and cut into small pieces. The tissue was distributed into

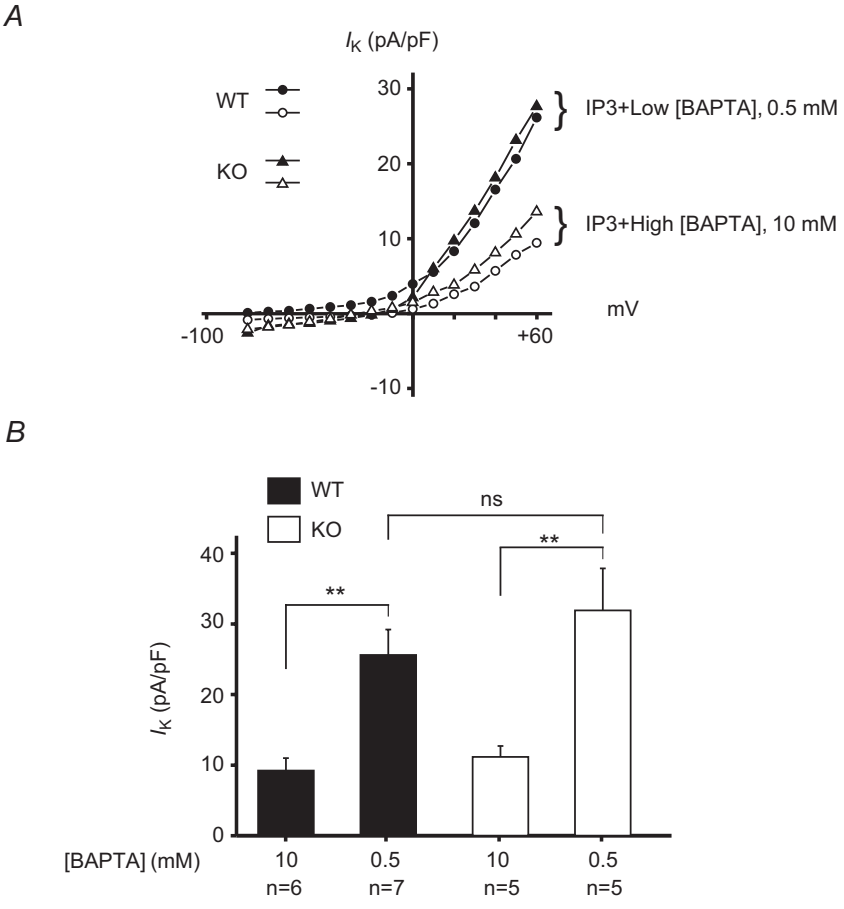

Figure 5. Calcium-activated K currents in lacrimal acinar cells
Patch-clamp recording of whole-cell currents was performed on single mouse lacrimal cells isolated from wild-type (WT) and Orai-KO (KO) mice. Cells were bathed in HBSS containing 1.2 mM $CaCl_2$. A whole-cell patch was established with an internal patch pipette solution containing 10 μM IP_3 with either 0.5 mM BAPTA (Low [BAPTA]) or 10 mM BAPTA (High [BAPTA]). Under low [BAPTA] conditions, IP_3 is able to mobilize and elevate intracellular Ca^{2+} and activate a K^+ current (I_K) in both WT and KO lacrimal cells. This effect is suppressed under high Ca^{2+} buffering conditions (High [BAPTA]). *A*, *I–V* relationships for I_K under both [BAPTA] conditions were indistinguishable between WT and KO lacrimal cells. *B*, current densities of I_K measured at +60 mV. Data are mean ± SEM (ANOVA; $^{**}P < 0.05$; n.s., not significant).

three cell strainers (15 mm Netwell Inserts, Corning, New York , USA) contained in a 12-well plate, and incubated in 2 ml of complete DMEM (10% fetal bovine serum, 5 mM glutamine) for 1 h at 37°C. The cell strainers containing lobules were then transferred and bathed in HBSS at 37°C containing (mM): NaCl 120, KCl 5.4, $MgCl_2$ 0.8, Hepes 20, $CaCl_2$ 1.8 and glucose 10; pH was adjusted to 7.4 with NaOH. HBSS was also supplemented with 0.3% (w/v) albumin. After 15 min, the samples were transferred into wells containing fresh HBSS (2 ml per well) for 30 min to establish a 'basal' secretion level. The samples were then transferred into wells containing 'test' solutions (2 ml) for an additional 30 min. The test solutions included: (i) control (HBSS + 1.8 mM $Ca^{2+}{}_o$), (ii) 200 μM MeCh + 1.8 mM $Ca^{2+}{}_o$ and (iii) 200 μM MeCh-$Ca^{2+}{}_o$ (+200 μM BAPTA).

On completion, the tissue and inserts were removed, and the bathing media (basal and test solutions) were assayed for peroxidase activity, an index of protein secretion. Peroxidase activity was measured using Amplex Red (Invitrogen, Carlsbad, CA, USA) (Rios *et al.* 2005) which, in the presence of hydrogen peroxide, is oxidized by the secreted peroxidase to produce a highly fluorescent molecule. The bathing solutions were incubated with 10 μM Amplex Red and 10 μM hydrogen peroxide and aliquoted in triplicate (150 μl) into the wells of a 96-well plate. The amount of fluorescence was then quantified simultaneously in each well on a $FLIPR^{TETRA}$ fluorescence imaging plate reader (Molecular Devices, Sunnyvale, CA, USA). Fluorescence was detected with a 470–495 nm excitation LED wavelength and a 565–625 nm emission filter. The effect of the test solutions on peroxidase secretion from the lacrimal gland pieces was expressed as a fold increase in fluorescence over that observed in the initial 30 min basal period (set as 1).

Results and discussion

We first confirmed by real-time RT-PCR knockdown of message for Orai1 in Orai1-KO mice (Fig. 1). We also determined message levels for other members of the Orai family (Orai2 and Orai3), TRPC1 – which has previously been shown to contribute to Ca^{2+} signalling in salivary glands (Liu *et al.* 2007) – and the two known Orai channel activators, STIM1 and STIM2. Orai1 message was essentially undetectable. Messages for Orai3, STIM1 and STIM2 were not affected by genotype. TRPC1 message increased about 2-fold but this was not statistically significant. Orai2 message increased substantially, as has been observed previously for other cell types from this mouse model (Vig *et al.* 2008). Interestingly, this increase in Orai2 message did not result in any detectable functional compensation with respect to SOCE, as shown in Fig. 2. Sustained Ca^{2+} entry due to thapsigargin was completely lost in lacrimal cells from KO mice as compared to their WT littermates (Fig. 2*A*–*D*). To determine whether loss of SOCE impacted the ability of acinar cells to maintain intracellular stores, we examined ionomycin-releasable Ca^{2+} as a measure of the intracellular stores. Treatment of mouse lacrimal cells with ionomycin (10 μM) in the absence of extracellular calcium produces a rapid release of Ca^{2+} such that the amplitude of the release transient is minimally affected by parallel Ca^{2+} buffering mechanisms. In unstimulated lacrimal cells, this ionomycin-sensitive store is essentially coincident with the agonist- and thapsigargin-sensitive stores (Bird *et al.* 1992*a*). The size of this ionomycin-releasable store was unchanged in cells from KO mice, indicating that intracellular Ca^{2+} stores were unchanged (Fig. 2*E* and *F*). Similar experiments utilizing the muscarinic-cholinergic agonist MeCh showed that the agonist-induced Ca^{2+} entry was also completely lost in the acinar cells from KO animals

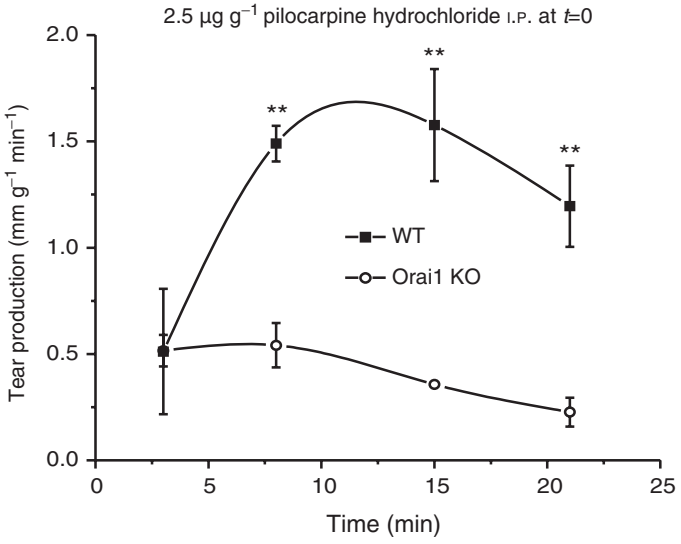

Figure 6. Pilocarpine-stimulated tear production in WT and Orai1-KO mice
Massive tear production was stimulated in anaesthetized mice by I.P. injection (at $t = 0$) of the muscarinic agonist pilocarpine (2.5 μg g^{-1} body weight in saline). Following injection, tear production was measured at the indicated time points using the phenol red string method, and adjusted for animal body weight (mm g^{-1} min^{-1}; see Methods). With comparisons made with paired littermates, tear production was significantly reduced in the Orai1-KO mice. Data are mean ± SEM from three sets of paired littermates (*t* test, $^{**}P < 0.05$).

(Fig. 3). We previously showed that high concentrations of muscarinic agonists produce sustained Ca^{2+} signals, while lower concentrations produce sinusoidal oscillations superimposed on an elevated basal Ca^{2+} level (Bird *et al.* 1993). The pattern of these oscillations varies somewhat from cell to cell, and in Fig. 3*C* we show the responses to low MeCh in three different WT and KO acinar cells. In all cases, whether saturating (Fig. 3*A*) or sub-saturating (Fig. 3*C*) concentrations of MeCh were used, the sustained Ca^{2+} signal ultimately failed in the KO acinar cells (summarized in Fig. 3*B*).

To ensure that the diminished Ca^{2+} signals did in fact result from a defect in SOCE, we also undertook measurement of the classical store-operated Ca^{2+} current, I_{CRAC} (Hoth & Penner, 1993; Parekh & Penner, 1997). However, as is often the case, Ca^{2+} currents clearly identifiable as I_{CRAC} could not be detected, perhaps owing to the small size of the current and to the substantial constitutive currents present in these cells. The leak currents were variable in magnitude, slightly outwardly rectifying, but were not significantly different in WT and KO cells (data not shown). In a previous study, a similar problem with small I_{CRAC} in the kidney cell line HEK293 was largely overcome by taking advantage of a property of I_{CRAC} whereby currents are rapidly and transiently increased by removal of extracellular divalent cations (Hoth & Penner, 1993; DeHaven *et al.* 2007). Figure 4*A* shows inward (at −120 mV) and outward (at +120 mV) whole cell currents in a lacrimal acinar cell patched with a pipet containing 25 μM IP_3 and 10 mM BAPTA to deplete intracellular Ca^{2+} stores. Inward Ca^{2+} current is not readily discernible, but a clear inwardly rectifying current is seen immediately upon switching the external medium to one that is free of divalent cations (Fig. 4*A* and *C*). This current is transient, owing to the property of de-potentiation, a hallmark of I_{CRAC} (Zweifach & Lewis, 1996). Fig. 4*B* shows a similar measurement on an acinar cell from an

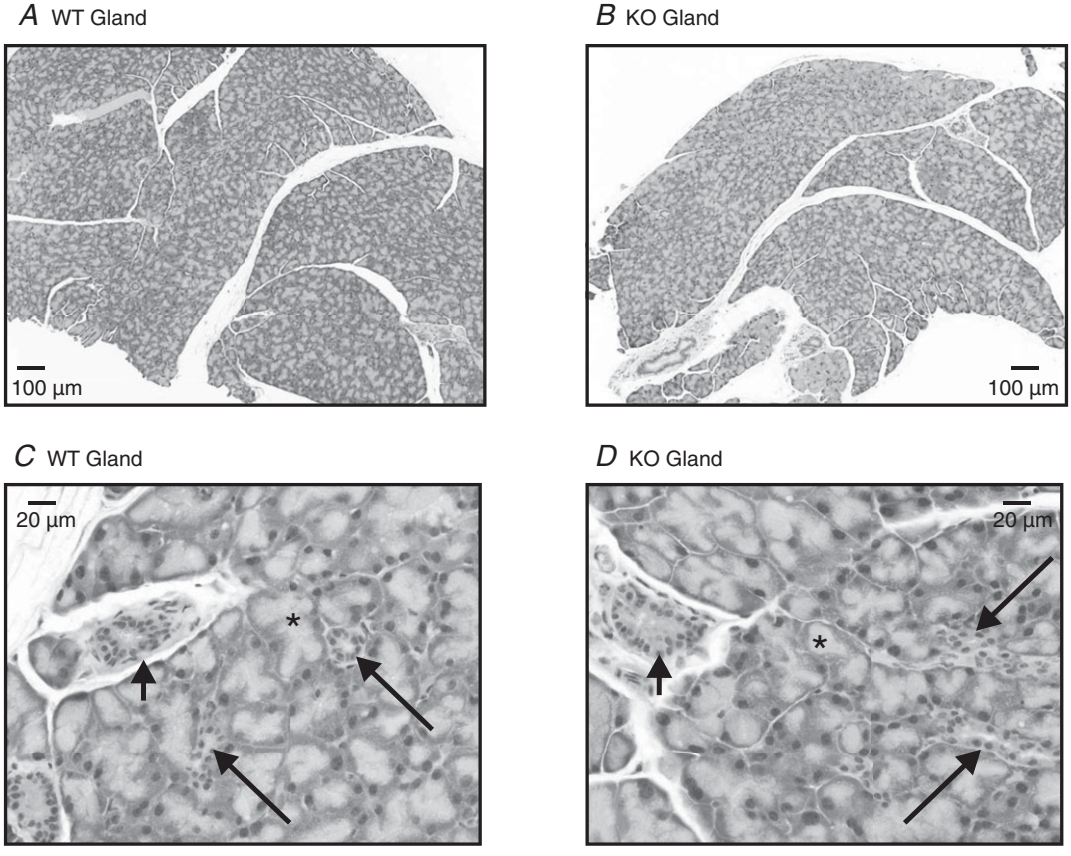

Figure 7. Histology of intact lacrimal glands
Excised lacrimal glands were harvested from wild-type (WT) and Orai1-KO (KO) mice, fixed in modified Karnovsky's fixative, embedded in paraffin and stained with H&E for histological examination as described in Methods. *A* and *C*, low magnification (4×) and a higher magnification (20×) of a representative lacrimal gland isolated from a WT mouse. *B* and *D*, low magnification (4×) and a higher magnification (20×) of a representative lacrimal gland isolated from an Orai1-KO mouse. WT and KO lacrimal glands were indistinguishable, composed of closely packed acini (asterisks) with interspersed intralobular ducts (long arrows) that convey the acinar secretions into the interlobular ducts (short arrows). Observations based on pairs of lacrimal glands isolated from five WT and KO mice (two male and three female mice used in each case).

Orai1-KO mouse. There is only a small inward current upon switching to a divalent cation medium, and this small current is mirrored in outward current, indicating that it is not inwardly rectifying and thus is not I_{CRAC}. Figure 4*D* summarizes leak-subtracted inward current measurements from multiple patch-clamp experiments with cells from WT and KO mice, demonstrating almost complete loss of divalent-free inward current in the KO animals.

To ensure that the loss of I_{CRAC} was not the result of a general loss of plasma membrane channels, and to determine whether other physiological components of the acinar ionic signalling pathway remain intact, we examined Ca^{2+}-activated K^+ currents, as described in Methods. Figure 5*A* shows the *I–V* relationship for outwardly rectifying K^+ currents in cells dialysed with IP_3 and 0.5 mM BAPTA, and that these currents are suppressed in the presence of a high (10 mM) concentration of BAPTA. Data from multiple experiments are summarized in Fig. 5*B*, showing that neither the constitutive nor the Ca^{2+}-activated currents are changed in acinar cells from KO mice.

We next determined the consequences of loss of SOCE in lacrimal cells to neurogenic secretion of tears in anaesthetized WT and KO mice by examining the flow of tears in response to the muscarinic–cholinergic agonist pilocarpine. As shown in Fig. 6, overflow tear production in WT mice continued at a substantial rate for over 20 min. In the KO mice, the rate of secretion was significantly less and all but ceased within a few minutes.

We noted that some but not all KO mice showed some signs of inflammation in their eyes. In an earlier report, using another global Orai1 knockout mouse, some degree of eyelid irritation was reported (Gwack *et al.* 2008). However, in a global knockout, it is not possible to determine if this results from diminished secretion or the compromised immunity expected in these mice. Furthermore, in a previous report utilizing mice with STIM1 and STIM2 deleted from T cells, disruption of salivary gland function was observed, and this was attributed to an increase in auto-immunity and pathological lymphocytic infiltration into the glands, leading ultimately to progressive salivary gland destruction. Thus, we next examined the structure of WT

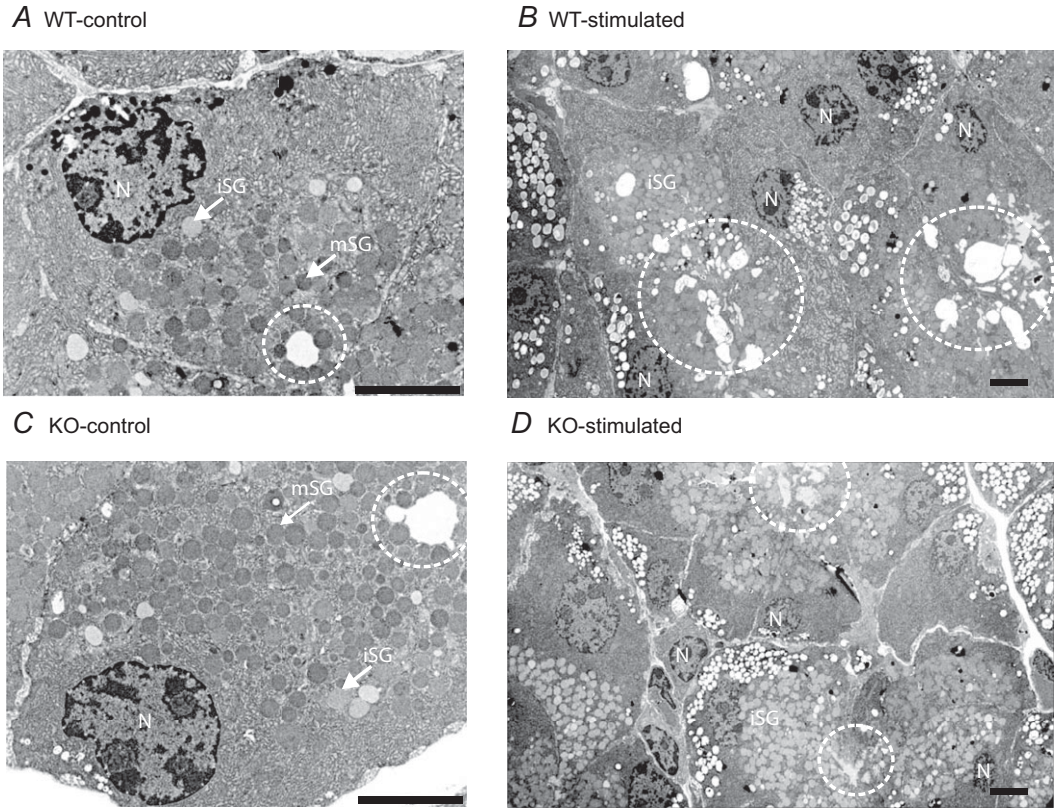

Figure 8. Transmission electron micrographs of lacrimal acini from wild-type (WT) and Orai1 knockout (KO) littermate mice under control and pilocarpine-stimulated conditions
A and *C*, representative electron micrographs of lacrimal tissue from control wild-type (WT-control) and Orai-KO (KO-control) mice, respectively. *B* and *D*, representative electron micrographs of lacrimal tissue from pilocarpine-stimulated (20 min), wild-type (WT-stimulated) and Orai-KO (KO-stimulated) mice respectively. Indicated on the micrographs are nuclei (N), immature secretory granules (iSG), mature secretory granules (mSG) and acinar lumens (dashed circle). Scale bars = 5 μm.

and KO lacrimal acinar cells by both light and electron microscopy. The general morphology of the glands and acinar cells was not notably different in WT and KO animals. As shown in Fig. 7, in both cases glands were composed of closely packed acini (asterisks), intralobular ducts (long arrows) and interlobular ducts (short arrows). At the electron microscopic level, Fig. 8 shows similar populations of immature and mature secretory granules (Fig. 8*A* and *C*, respectively), and following stimulation with the muscarinic–cholinergic agonist pilocarpine, both WT and KO animals showed signs of exocytotic secretion (Fig. 8*B* and *D*). No ductal abnormalities were noted in either the WT or KO glands. No consistent or widespread infiltrates of lymphocytes were observed in the glands of either the WT or the KO animals, nor was there any evidence of destructive glandular changes, similar to those described by Cheng *et al.* (2012). Electron microscopy revealed evidence of a few inflammatory cells (lymphocytes); however, these cells were few in number and were not found in acinar lumens but most often within the surrounding ducts or connective tissue. There was no gland destruction at all, especially destruction due to lymphocyte infiltrates noted by electron microscopy.

We further explored the consequences of loss of SOCE in lacrimal glands to neurogenic stimulation in response to stimulation. The morphometry of lacrimal acinar lumens under control and pilocarpine-stimulated (20 min) conditions was analysed blindly to generate a 'secretion index' based on three characteristics: lumen dilatation, presence of membrane fragments and empty secretory granules (Fig. 9*A*). Cholinergic stimulation resulted in a significant increase in the secretory index for both WT and Orai1-KO lacrimal tissue. However, the secretory index was significantly less in pilocarpine-treated Orai1-KO tissue than in the WT tissue (Fig. 9*A*). To confirm the decreased protein secretion, we carried out assays of agonist-induced release of peroxidase, a major secretory protein in rodent exorbital lacrimal glands (Dartt, 1989; Rios *et al.* 2005). As shown in Fig. 9*B*, the muscarinic cholinergic agonist MeCh induced a significant increase in the release of peroxidase from lacrimal gland fragments. This release of peroxidase was reduced, although not entirely blocked, by removal of extracellular

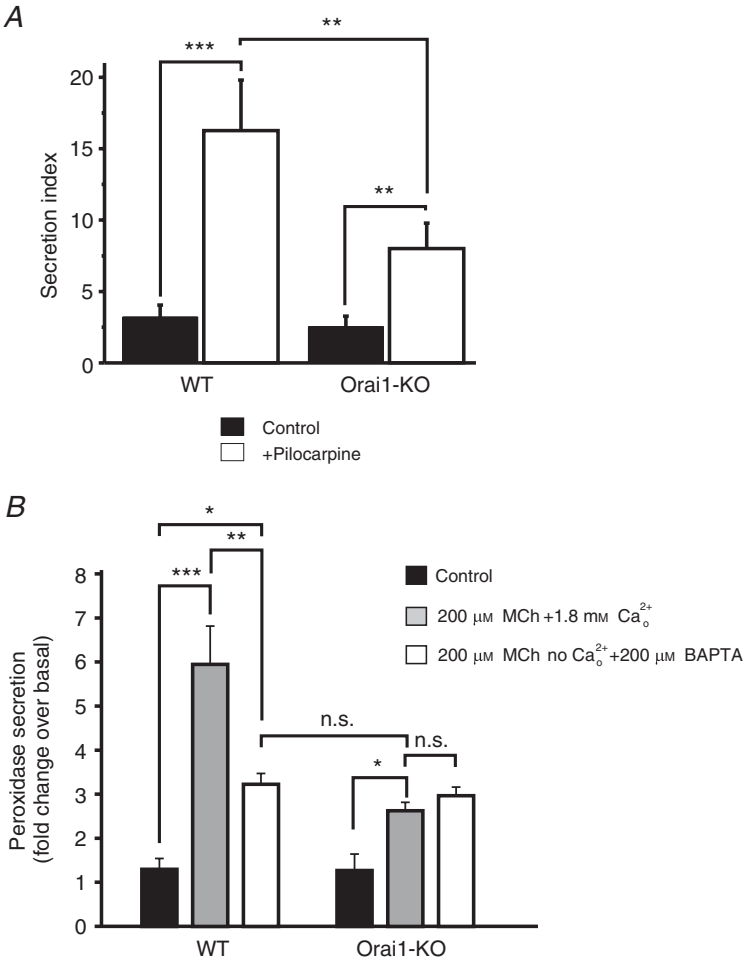

Figure 9. The effects of muscarinic stimulation on protein secretion in lacrimal tissue
A, comparison of 'secretion index' (see Methods) from morphometric analysis of electron micrographs of lacrimal acini (as in Fig. 8). In total, 20 electron micrographs were analysed from each mouse group, which numbered: six WT-control, six KO-control, four WT-stimulated and four KO-stimulated. Data are mean ± SEM as described in Methods (***$P < 0.0001$; **$P < 0.05$). *B*, effects of MeCh on peroxidase secretion from lacrimal gland pieces isolated from wild-type (WT) and Orai1-KO (KO) mice. The effect of MeCh was monitored both in the presence and absence of extracellular calcium (1.8 mM). The data are mean ± SEM from four sets of paired littermates (ANOVA; ***$P < 0.0001$; **$P < 0.01$; *$P < 0.05$; n.s., not significant).

Ca^{2+}. The residual Ca^{2+}-independent secretion could result either from the transient release of intracellular Ca^{2+} or possibly from a contribution of the protein kinase C pathway (Dartt, 1989). Importantly, when secretion was measured in fragments from KO mice, the release of peroxidase was indistinguishable in the presence or absence of Ca^{2+}, and was similar to that seen in the absence of Ca^{2+} with fragments from WT animals. These morphological and biochemical data demonstrate, first, that knockdown of Orai1 and loss of SOCE has minimal effect on the development and differentiation of lacrimal glands, and, second, that cholinergic stimulated secretion (Ding *et al.* 2003) occurs both in WT and, to a lesser extent, in Orai1-KO mouse lacrimal glands. The lack of change in the Ca^{2+}-independent peroxidase secretion is consistent with the morphological analyses indicating that the glands from KO animals lack SOCE, but have no other non-specific alterations in lacrimal gland function

Lacrimal glands from mice or rats have long served as useful models for basic studies of Ca^{2+} signalling (Putney & Bird, 1994). For example, rat lacrimal gland studies provided early evidence that Ca^{2+} entry could be activated independently of receptor activation (Parod & Putney, 1978). One of the earliest demonstrations of the ability of thapsigargin to activate SOCE was carried out with lacrimal acinar cells (Kwan & Putney, 1990). In recent years, attention has turned to haematopoietic cells because of the ability to measure the current underlying SOCE, I_{CRAC} (Hoth & Penner, 1992). It has traditionally been difficult to measure I_{CRAC} in non-haematopoetic cells because of its extremely small size; here we utilize a technique whereby I_{CRAC} is substantially amplified by removal of divalent cations (Hoth & Penner, 1993; DeHaven *et al.* 2007) to provide the first demonstration of an I_{CRAC}-like current in this cell type. In addition, the results of this study demonstrate the necessary role of SOCE, involving channels comprising Orai1 subunits, in Ca^{2+} signalling and function of mouse lacrimal glands.

As mentioned above, a strategy that compromises SOCE in T cells also leads to a Sjögren's syndrome-like diminished exocrine secretion, through an increase in autoimmunity (Cheng *et al.* 2012). Thus, SOCE obviously impacts on exocrine gland function at both the local level, as shown here, and the level of the immune system. We can speculate that alleviation of debilitating exocrinopathies, such as dry eye syndrome, may involve modulation of SOCE or other Ca^{2+} entry pathways.

References

Bird GS, Rossier MF, Obie JF & Putney JW (1993). Sinusoidal oscillations in intracellular calcium requiring negative feedback by protein kinase C. *J Biol Chem* **268**, 8425–8428.

Bird GStJ, Obie JF & Putney JW (1992*a*). Functional homogeneity of the non-mitochondrial Ca^{2+}-pool in intact mouse lacrimal acinar cells. *J Biol Chem* **267**, 18382–18386.

Bird GStJ, Obie JF & Putney JW (1992*b*). Sustained Ca^{2+} signalling in mouse lacrimal acinar cells due to photolysis of 'caged' glycerophosphoryl-*myo*-inositol 4,5-bisphosphate. *J Biol Chem* **267**, 17722–17725.

Cahalan MD, Zhang SL, Yeromin AV, Ohlsen K, Roos J & Stauderman KA (2007). Molecular basis of the CRAC channel. *Cell Calcium* **42**, 133–144.

Cheng KT, Alevizos I, Liu X, Swaim WD, Yin H, Feske S, Oh-hora M & Ambudkar IS (2012). STIM1 and STIM2 protein deficiency in T lymphocytes underlies development of the exocrine gland autoimmune disease, Sj+¦gren's syndrome. *Proc Natl Acad Sci U S A* **109**, 14544–14549.

Dartt DA (1989). Signal transduction and control of lacrimal gland protein secretion: a review. *Current Eye Res* **8**, 619–636.

Dartt DA (2004). Dysfunctional neural regulation of lacrimal gland secretion and its role in the pathogenesis of dry eye syndromes. *Ocul Surf* **2**, 76–91.

Dartt DA (2009). Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. *Prog Retin Eye Res* **28**, 155–177.

Davis FM, Parsonage MT, Cabot PJ, Parat MO, Thompson EW, Roberts-Thomson SJ & Monteith GR (2013). Assessment of gene expression of intracellular calcium channels, pumps and exchangers with epidermal growth factor-induced epithelial-mesenchymal transition in a breast cancer cell line. *Cancer Cell Int* **13**, 76.

DeHaven WI, Smyth JT, Boyles RR & Putney JW (2007). Calcium inhibition and calcium potentiation of Orai1, Orai2, and Orai3 calcium release-activated calcium channels. *J Biol Chem* **282**, 17548–17556.

Ding C, Walcott B & Keyser KT (2003). Sympathetic neural control of the mouse lacrimal gland. *Invest Ophthalmol Vis Sci* **44**, 1513–1520.

Dursun D, Wang M, Monroy D, Li DQ, Lokeshwar BL, Stern ME & Pflugfelder SC (2002). A mouse model of keratoconjunctivitis sicca. *Invest Ophthalmol Vis Sci* **43**, 632–638.

Godfrey PP & Putney JW (1984). Receptor-mediated metabolism of the phosphoinositides and phosphatidic acid in rat lacrimal acinar cells. *Biochem J* **218**, 187–195.

Gwack Y, Srikanth S, Oh-Hora M, Hogan PG, Lamperti ED, Yamashita M, Gelinas C, Neems DS, Sasaki Y, Feske S, Prakriya M, Rajewsky K & Rao A (2008). Hair loss and defective T- and B-cell function in mice lacking ORAI1. *Mol Cell Biol* **28**, 5209–5222.

Hogan PG, Lewis RS & Rao A (2010). Molecular basis of calcium signalling in lymphocytes: STIM and ORAI. *Annu Rev Immunol* **28**, 491–533.

Hoth M & Penner R (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* **355**, 353–355.

Hoth M & Penner R (1993). Calcium release-activated calcium current in rat mast cells. *J Physiol* **465**, 359–386.

Kwan CY & Putney JW (1990). Uptake and intracellular sequestration of divalent cations in resting and methacholine-stimulated mouse lacrimal acinar cells. Dissociation by Sr^{2+} and Ba^{2+} of agonist-stimulated divalent cation entry from the refilling of the agonist-sensitive intracellular pool. *J Biol Chem* **265**, 678–684.

Kwan CY, Takemura H, Obie JF, Thastrup O & Putney JW (1990). Effects of methacholine, thapsigargin and La^{3+} on plasmalemmal and intracellular Ca^{2+} transport in lacrimal acinar cells. *Am J Physiol* **258**, C1006–C1015.

Liu X, Cheng KT, Bandyopadhyay BC, Pani B, Dietrich A, Paria BC, Swaim WD, Beech D, Yildrim E, Singh BB, Birnbaumer L & Ambudkar IS (2007). Attenuation of store-operated Ca^{2+} current impairs salivary gland fluid secretion in TRPC1(/) mice. *Proc Natl Acad Sci U S A* **104**, 17542–17547.

Parekh AB & Penner R (1997). Store depletion and calcium influx. *Physiol Rev* **77**, 901–930.

Parod RJ & Putney JW (1978). The role of calcium in the receptor mediated control of potassium permeability in the rat lacrimal gland. *J Physiol* **281**, 371–381.

Putney JW (1986). A model for receptor-regulated calcium entry. *Cell Calcium* **7**, 1–12.

Putney JW & Bird GS (1994). The inositol phosphate-calcium signalling system in lacrimal gland cells. *Adv Exp Med Biol* **350**, 115–119.

Putney JW & Bird GStJ (1998). Calcium signalling in lacrimal acinar cells. *Adv Exp Med Biol* **438**, 123–128.

Rios JD, Horikawa Y, Chen LL, Kublin CL, Hodges RR, Dartt DA & Zoukhri D (2005). Age-dependent alterations in mouse exorbital lacrimal gland structure, innervation and secretory response. *Exp Eye Res* **80**, 477–491.

Suchanek KM, May FJ, Robinson JA, Lee WJ, Holman NA, Monteith GR & Roberts-Thomson SJ (2002). Peroxisome proliferator-activated receptor α in the human breast cancer cell lines MCF-7 and MDA-MB-231. *Mol Carcinog* **34**, 165–171.

Trautmann A & Marty A (1984). Activation of Ca-dependent K channels by carbamoylcholine in rat lacrimal glands. *Proc Natl Acad Sci U S A* **81**, 611–615.

Vig M, DeHaven WI, Bird GS, Billingsley JM, Wang H, Rao PE, Hutchings AB, Jouvin MH, Putney JW & Kinet JP (2008). Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels. *Nat Immunol* **9**, 89–96.

Zweifach A & Lewis RS (1996). Calcium-dependent potentiation of store-operated calcium channels in T lymphocytes. *J Gen Physiol* **107**, 597–610.

Additional information

Competing interests

All authors ascertain no conflict of interests associated with this work.

Author contributions

J.X.: carried out experiments providing published data; conception and design, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content. J.G.P.: assisted in experiments providing published data; conception and design, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content. F.M.D.: carried out experiments providing published data; conception and design, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content. P.N.D.: carried out experiments providing published data; conception and design, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content. J.W.P.: conception and design, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content. G.S.B.: carried out experiments providing published data; conception and design, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content. All authors gave final approval of the version to be published.

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