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Calcium Signaling in Lacrimal Glands

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Abstract

Lacrimal glands provide the important function of lubricating and protecting the ocular surface. Failure of proper lacrimal gland function results in a number of debilitating dry eye diseases. Lacrimal glands secrete lipids, mucins, proteins, salts and water and these secretions are at least partially regulated by neurotransmitter-mediated cell signaling. The predominant signaling mechanism for lacrimal secretion involves activation of phospholipase C, generation of the Ca^{2+}-mobilizing messenger, IP_3, and release of Ca^{2+} stored in the endoplasmic reticulum. The loss of Ca^{2+} from the endoplasmic reticulum then triggers a process known as store-operated Ca^{2+} entry, involving a Ca^{2+} sensor in the endoplasmic reticulum, STIM1, which activates plasma membrane store-operated channels comprised of Orai subunits. Recent studies with deletions of the channel subunit, Orai1, confirm the important role of SOCE in both fluid and protein secretion in lacrimal glands, both *in vivo* and *in vitro.*

Introduction

The major function of lacrimal glands is to provide water, electrolytes, proteins and mucins to lubricate and protect the environmentally exposed surfaces of the eye (cornea and conjunctiva) [1]. Mammals have a major gland associated with each eye, and a number of minor glands (i.e., goblet cells, meibomian gland), which contribute to constitutive and neurogenic tears and all of which may be involved in pathological conditions when functionally impaired. An understanding of the basic mechanisms underlying lacrimal gland secretion may provide insights to the treatment of debilitating age-related dry eye diseases, as well as the more general exocrine dysfunction in Sjögren's syndrome [2]. Here we review the basic cell biology underlying the signaling pathways leading to secretion of proteins and fluid from the major lacrimal glands.

The flow of tears has long been known to be under both parasympathetic and sympathetic control [1;3;4]. Early studies demonstrated that stimulation of muscarinic-cholinergic receptors increased the discharge of granule stored protein, largely peroxidase, from rat

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exoribital lacrimal gland [5-7]. While not fully defined, the cholinergic-induced calcium signal likely exerts multiple effects during protein secretion to coordinate the mobilization of secretory vesicles to the lacrimal apical membrane where they fuse and release their contents. Synaptotagmin is a likely target to detect the cholinergic-induced calcium signal which works in concert with SNARE proteins (including VAMP8) to complete and open the vesicle fusion pore [8].

Ca^{2+}-dependent activation of monovalent channels (K^- and Cl^-) plays a critical role in fluid secretion, generating electrochemical and osmotic gradients to drive the movement of water and accumulation of electrolytes into the lumen of acinar clusters and the ductal system [9]. In *in vitro* preparations, muscarinic receptors increased the efflux of K^+ [7], a response thought to reflect ionic movements related to fluid secretion. Both protein secretion and K^+ efflux responses depended at least partially upon extracellular Ca^{2+}, and were associated with increased uptake of radioactive Ca^{2+} into the glands [6;7].

Subsequently, it was demonstrated that an α-adrenoceptor mechanism similarly activated both protein discharge and increased K^+ permeability [10-12], although curiously the α-adrenergic protein secretion was somewhat less sensitive to removal of extracellular Ca^{2+}. Unlike parotid salivary glands, lacrimal glands do not apparently contain β-adrenoceptors, but do contain adenylyl cyclase activating vasoactive intestinal peptide receptors [13] and melanotropin receptors [14]. Other Ca^{2+}-linked receptor types shown to significantly modulate lacrimal secretion include multiple types of purinergic P2X and P2Y receptors [15-18], substance P, serotonin, histamine [19] and protease-activated receptors [20].

In early studies of Ca^{2+} signaling, direct measurement of intracellular Ca^{2+} with chemical or genetically encoded indicators was not available. Changes in intracellular Ca^{2+} were often inferred from the time course and magnitude of Ca^{2+}-mediated responses, and for lacrimal glands and certain other epithelial cells, Ca^{2+}-activated K^+ channels provided this link [21]. The rate of K^+ efflux from lacrimal cells was assessed by an isotope washout technique whereby slices of lacrimal gland were equilibrated with $^{86}Rb^+$, a surrogate for K^+ [22]. By stepwise transfer of the slices through a series of non-radioactive incubations, released radioactivity could be measured and time-based changes in the first order rate constant for $^{86}Rb^+$ efflux calculated. By use of an experimental sequence first described for studies in parotid gland [23;24], protocols omitting and restoring extracellular Ca^{2+} revealed a biphasic response: the initial transient increase in $^{86}Rb^+$ efflux was independent of extracellular Ca^{2+}, while the sustained efflux response depended on extracellular Ca^{2+} being present [25] (Figure 1). The Ca^{2+} independent component of the response was thought to result from an intracellular release of Ca^{2+}, because only one such response could be obtained in the absence of external Ca^{2+}, and then an incubation in Ca^{2+}-containing medium was necessary to restore the response. This latter finding will be discussed in more detail in relation to its relevance to the mechanism of Ca^{2+} influx.

Release of intracellular Ca^{2+}

Ca^{2+} signaling in lacrimal acinar cells was initially seen to result from a biphasic mobilization of Ca^{2+} to the cytoplasm, an initial release of intracellular Ca^{2+} which was

followed by or accompanied by an increase in Ca^{2+} entry across the plasma membrane [7]. The intracellular release mechanism was the first to be solved. From as early as the 1950's, it was known that certain receptors, including muscarinic cholinergic receptors, stimulated a turnover of inositol lipids [26]. In 1975, Bob Michell [27] published his classic review on inositol lipids in which he proposed that this turnover in some manner served to link receptor activation to Ca^{2+} signaling. In 1983, Mike Berridge demonstrated that following receptor activation, the head group of phosphatidylinositol 4,5-bisphosphate, inositol 1,4,5-trisphosphate (IP_3), rapidly appeared in fly salivary glands, and suggested that this molecule served as a second messenger for Ca^{2+} release [28]. Soon thereafter, in a collaboration between Berridge, Irene Schulz and Robin Irvine, IP_3 was shown to release Ca^{2+} from non-mitochondrial stores in a preparation of permeabilized pancreatic acinar cells [29]. Consistent with this idea, in lacrimal glands Ca^{2+}-mobilizing agonists stimulated turnover of inositol lipids and this involved degradation of phosphatidylinositol 4,5-*bis*phosphate and formation of soluble inositol phosphates [30]. IP_3 was later shown to release intracellular Ca^{2+} in lacrimal acinar cells, by a technique involving introduction of the molecule in intact acinar cells via a patch pipet [31]. IP_3 has also been shown to release intracellular Ca^{2+} in permeabilized lacrimal acinar cells [32], and following microinjection into lacrimal acinar cells [33]. This release of Ca^{2+} appears to come from a relatively homogenous pool of Ca^{2+} within the endoplasmic reticulum. Thus, in permeabilized cell experiments in other exocrine glands, inhibition of mitochondrial uptake of Ca^{2+} does not impair loading of the pool sensitive to IP_3 [29]. Interestingly, spatial measurement of acetylcholine-induced Ca^{2+} signals in clusters of rat lacrimal cells demonstrate a distinct gradient of $[Ca^{2+}]_i$ that appears to be maximal at the luminal pole of the cell [34]. Thus, while the agonist-sensitive Ca^{2+} signal appears to be released from a homogeneous ER Ca^{2+} pools, the spatial characteristics of the Ca^{2+} signal may be determined by $InsP_3$ receptors localized to specific regions of the cell. This pattern of calcium release may result in differential physiological effects at luminal versus basolateral membranes, for example in control of lacrimal secretion.

As will be discussed below, a useful tool for studying Ca^{2+} pools is the plant toxin, thapsigargin, that inhibits the endoplasmic reticulum Ca^{2+} pump (SERCA) and specifically releases endoplasmic reticulum Ca^{2+} [35]. In permeabilized lacrimal acinar cells, prior discharge of thapsigargin-sensitive Ca^{2+} stores precluded any further release by IP_3, confirming that the source in lacrimal cells is the endoplasmic reticulum. The homogeneity of this pool was demonstrated in a study utilizing fura-2-loaded attached primary mouse lacrimal acinar cells [36]. Intracellular stores were discharged, in a Ca^{2+} depleted medium, by one of three agents: methacholine, presumed to release the IP_3-sensitive pool; thapsigargin, which would release the total endoplasmic reticulum pool; and the calcium ionophore, ionomycin, which would discharge essentially all intracellular Ca^{2+} pools. Each of these three strategies essentially prevented further release by either of the other two. For example, after Ca^{2+} release by methacholine, not further release was seen with either thapsigargin or ionomycin. However, when Ca^{2+} was elevated for a prolonged period, with a high concentration of methacholine, a pool of Ca^{2+} appeared in excess of that which could be released by thapsigargin. Loading of this pool was prevented by injection of the mitochondrial Ca^{2+} uptake inhibitor, ruthenium red. Thus, consistent with other studies, the

mitochondria contain little Ca^{2+} at rest, but actively accumulate it when it is released by IP_3 [37].

IP_3 releases Ca^{2+} from the endoplasmic reticulum by activating a receptor-ion channel, the IP_3 receptor. The receptor was first described by Spät *et al.* [38] in permeabilized hepatocytes and was cloned by Mikoshiba [39], who subsequently described three gene products termed types 1, 2 and 3 IP_3 receptor [40]. Knockout in mice of the type 1 receptor produces a severe ataxia, but double knockout of the types 2 and 3 results in an exocrine secretion deficit and pups become malnourished [41]. In that same animal model, double knockout of types 2 and 3 IP_3 receptor also reduces salivary gland amylase secretion. With evidence of all three IP_3 receptor types expressed in mouse lacrimal tissue [42], it will be interesting to study the consequences of their knockdown on lacrimal gland function.

Calcium oscillations in lacrimal acinar cells and feedback regulation of signaling

In many cell types utilizing phospholipase C-mediated Ca^{2+} signaling, low physiological concentrations of agonists do not produce sustained Ca^{2+} signals, as shown in Figure 1, but rather bring about a complex pattern of cytoplasmic Ca^{2+} transients termed Ca^{2+} oscillations [43;44]. The shapes and properties of these oscillations can vary depending upon cell type and the nature of the agonist. In some cases, agonists acting on different phospholipase C-linked receptors, but in the same cell type, can produce oscillations with markedly different properties [45]. The two most likely contributors to these oscillatory behaviors are feedback regulation of the Ca^{2+} release mechanism, producing oscillations in Ca^{2+} release at a constant level of IP_3, and feedback regulation of steps upstream of phospholipase C resulting in oscillating production of IP_3 [44]. When oscillations involve regenerative activation mechanisms, they are generally of constant magnitude (all-or-none) and vary in frequency as a function of the stimulus strength (agonist concentration). However, in lacrimal acinar cells, precisely the opposite is seen; in this cell type, oscillations in response to muscarinic receptor activation occur on an elevated basal level of Ca^{2+} and are relatively constant in frequency at different agonist concentrations (Figure 2) [46]. With increasing agonist concentration, the average cytoplasmic concentration rises until at near saturating concentrations the oscillations disappear and the cells respond with a sustained elevation as in Figure 1.

Oscillations of this nature, of relatively constant frequency, would likely involve only a simple negative feedback mechanism the time constant of which is slower than the processes regulating cytoplasmic Ca^{2+}. In lacrimal acinar cells, this feedback mechanism appears to be protein kinase C [46]. The oscillations are completely lost when protein kinase C is either strongly activated or inhibited [46]. There is considerable evidence that protein kinase C can serve as a regulator of G-protein coupled receptors [47;48], and in lacrimal acinar cells, activation of protein kinase C by phorbol esters strongly inhibits Ca^{2+} signaling, IP_3 production, but does not inhibit Ca^{2+} signaling due to direct injection of IP_3 into acinar cells [46].

The physiological significance of frequency modulated, baseline Ca^{2+} spikes is widely appreciated, the general consensus being that such a pattern can produce a digital signal with high signal-to-noise [49;50]. However, for the muscarinic receptor-mediated Ca^{2+} signals in lacrimal cells, the constant frequency oscillations are only seen when experiments are carried out at room temperature, likely due to a strong temperature dependence of protein kinase C. They thus have little functional importance as oscillations *per se*, yet understanding their mechanism reveals an important feedback regulator of Ca^{2+} signaling in this cell type. Since muscarinic receptor activation in exocrine gland undoubtedly occurs through localized acetylcholine release from nearby parasympathetic nerves, it is possible that digitized signaling results from the magnitude and timing of neurosecretion at parasympathetic-acinar cell connections.

Ca^{2+} entry

The Ca^{2+} entry phase of Ca^{2+} signaling in lacrimal glands is believed to involve the process of *capacitative calcium entry* or *store-operated calcium entry* (SOCE) [51;52]. Some of the origins of this concept lie in early experiments carried out with lacrimal gland cells (or slices). A key experiment was a variation on the one already mentioned that established that the first phase of $^{86}Rb^{+}$ release was due to intracellular Ca^{2+} release. In order to refill the intracellular Ca^{2+} store following its discharge in a Ca^{2+} depleted medium, it was necessary to briefly incubate slices in a medium containing Ca^{2+}. This experiment was first carried out with parotid gland, but in this case, owing perhaps to a prejudice as to how the Ca^{2+} would enter the cells, the “loading” in extracellular Ca^{2+} was tested while agonist receptors were occupied [24]. The logic was that the Ca^{2+} would need the receptor-activated channels to be open in order to enter the cell and refill the store. However, in a subsequent study with lacrimal gland, a control was added to the experiment: Ca^{2+} was also briefly added to the preparation after blocking the receptor [25]. Surprisingly, the intracellular stores loaded just as completely whether the receptor was occupied or not (Figure 3). This provided the first experimental evidence that it was not a direct receptor-dependent signal that kept the channels open, but rather they remained open as long as the intracellular stores were empty.

Eventually, this and other pieces of circumstantial evidence would lead to the formalization of the capacitative model [51]. When first described, there was some uncertainty as to whether the loading of intracellular Ca^{2+} stores occurred by some discrete route bypassing the cytoplasm [53], or through a series of membrane permeations followed by uptake into the endoplasmic reticulum. A number of experimental observations accumulated to support the latter view. Perhaps the clearest, and most important for many other reasons was the action of the SERCA inhibitor, thapsigargin [54]. Thapsigargin was shown to quantitatively recapitulate agonist-induced Ca^{2+} entry, first in parotid gland [54], and subsequently in lacrimal gland [32]. According to the direct reloading model, influx into the cytoplasm would only occur following continuous release from the endoplasmic reticulum. In the case of exocrine glands, this would be through the activated IP_3 receptor. However, thapsigargin emptied intracellular endoplasmic reticulum stores, but did not increase cellular IP_3 levels [32;54]. Thus, the rate of permeation of Ca^{2+} into the cytoplasm was unrelated to the permeability of the endoplasmic reticulum to Ca^{2+}, and Ca^{2+} must come to the cytoplasm directly via store-operated plasma membrane Ca^{2+} channels. Consistent with this

conclusion, soon thereafter a transmembrane electrophysiological Ca^{2+} current underlying SOCE was described in mast cells, termed I_{crac} for calcium-release-activated calcium current [55].

With the exception of hematopoietic cells, I_{crac} is often too small to detect when Ca^{2+} is the charge carrier. It can be detected, however, by exploiting a property such that the deletion of all external divalent cations removes its divalent selectivity permitting Na^+ to permeate, and thereby giving substantially larger and readily detectable currents [56]. An inwardly rectifying Na^+ current, under conditions of Ca^{2+} store depletion and a divalent cation free external solution was recently described for mouse lacrimal acinar cells [57].

Inositol tetrakisphosphate and Ca^{2+} entry in lacrimal acinar cells

Inositol 1,4,5-trisphosphate is formed when agonists, through either a G-protein dependent or tyrosine kinase dependent mechanism, activate a phospholipase C to cleave the head group from phosphatidylinositol 4,5-bisphosphate. IP_3 is then rapidly metabolized by two enzymes. A 5-phosphatase cleaves the phosphate from position 5 of the inositol ring, giving inositol 1,4-bisphosphate which is incapable of releasing Ca^{2+}. A 3-kinase phosphorylates IP_3 at the 3 position, resulting in the formation of inositol 1,3,4,5-tetrakisphosphate (IP_4) [58]. The rapidity of formation of IP_4 led to the suggestion that it might have some signaling function distinct from that of IP_3, specifically, the activation of the second, Ca^{2+} entry phase of signaling [59]. This idea was examined by experiments in which IP_3, IP_4 or a combination of the two was dialyzed into lacrimal acinar cells by patch pipet perfusion, while Ca^{2+} changes were assessed from outward K^+ currents known to be Ca^{2+}-activated [31]. Perfusion of the cells with IP_3 produced variable results ranging from no response to rapidly inactivating transient responses. Perfusion with IP_4 caused no effect when used alone. However, inclusion of both IP_3 and IP_4 in the pipet resulted in robust and sustained increases in K^+ conductance ($[Ca^{2+}]_i$). The authors interpreted this result as indicating a role for IP_4 in the sustained, Ca^{2+} entry component of lacrimal acinar cell signaling [31]. However, in a subsequent publication, the same group showed that in fact IP_4 could also augment the ability of IP_3 to release Ca^{2+} [60]. IP_4 is a substrate for the same 5-phosphatase that metabolizes IP_3, and has a lower Km but a slow turnover rate [61]. Thus, IP_4 would efficiently block the metabolism of IP_3, and this could explain the effects of IP_4 in the patch perfusion experiments; i.e., the sustained response requires sustained depletion of stores by IP_3, and IP_4 allows this by protecting IP_3 from the 5-phosphatase [62]. In support of this interpretation, perfusion by patch pipet, or injection into intact lacrimal cells of a non-metabolizable but fully efficacious isomer of IP_3, (2,4,5)IP_3, fully activated sustained Ca^{2+} entry, whether measured as Ca^{2+}-activated K^+ conductance, or by use of the Ca^{2+} indicator, Fura-2 [63]. The interpretation was that the effect of IP_4 in the previous studies was indeed likely due to protection of IP_3 from metabolism. A subsequent publication demonstrated this by more direct measurements of the interactions of IP_3 and IP_4 [62].

Lacrimal secretion in an Orai1 knockout mouse

Throughout the 1990's and 2000's, considerable research focused on searching for candidates for the signal that activates store-operated channels, and for the channel itself

(see numerous examples in [64]). A number of reports suggested the presence of a diffusible messenger, termed "CIF" for calcium influx factor [65;66]. With regard to the channel, much attention was focused on TRPC channels, which are clearly activated by phospholipase C-dependent mechanisms, and can pass considerable Ca^{2+} [67]. Although still somewhat controversial, it appears that at least a component of the mechanism for activating TRPC channels, under some conditions, can involve depletion of endoplasmic reticulum stores [68;69]. However, TRPC channels clearly do not share the biophysical properties of I_{crac}. Nonetheless, knockdown or knockout of specific TRPC channels has been shown to impair exocrine secretion in salivary glands [70] and pancreas [71].

The major molecular components of I_{crac}, STIM1 and Orai1, were discovered by a series of targeted and whole-genome RNAi screens [72]. STIM1 (or STIM2 under some circumstances), serves as the Ca^{2+} sensor in the endoplasmic reticulum. STIM1 is a single pass membrane spanning protein which contains a Ca^{2+}-binding (and Ca^{2+} sensing) EF-hand in the lumenally-directed N-terminus. Loss of Ca^{2+} from the endoplasmic reticulum results in dissociation of Ca^{2+} from STIM1, aggregation of STIM1, and accumulation of STIM1 in junctions between endoplasmic reticulum and the plasma membrane [73;74]. There, STIM1 can bind to and activate store-operated channels comprised of Orai1 subunits [75]. Mammals also express two other Orai proteins, Orai2 and Orai3 [76], whose functions are less well understood (but see [77]).

Mice lacking Orai1 tended to die perinatally, presumably due to compromised skeletal muscle development [78;79], but some pups survive with special housing conditions [78], or when the mice are crossed into an outbred strain [79]. The lacrimal glands of Orai1 knockout mice appeared to develop normally, but the secretion *in vivo* of cholinergically-induced overflow tears was substantially curtailed [57]. *In vitro*, agonist-activated protein (peroxidase) secretion was reduced to the level seen in the absence of external Ca^{2+}. Sustained Ca^{2+} entry, whether due to a cholinergic agonist or thapsigargin, was essentially absent. Quantitative PCR demonstrated that of the known SOCE mediators, only Orai1 message was decreased (essentially gone), while message for Orai3 and for STIM1 and STIM2 were not statistically changed. Interestingly, message for Orai2 was substantially increased, yet this failed to compensate for the loss of Orai1, as SOCE was not detectable. The store-operated current, I_{crac}, measured as a Na^+ current under divalent-free conditions (see above), was also lost in the knockout mice.

In an earlier report, T-cell specific knockout of both STIM1 and STIM2 resulted in a Sjögren's syndrome-like condition such that salivary glands degenerated due to an increased autoimmunity and extensive lymphocytic invasion [80]. Orai1 knockout mice would be expected to have compromised T-cell function as well, but these mice showed no evidence of glandular degeneration or lymphocytic invasion [57]. Significantly, the component of protein secretion that did not depend on external Ca^{2+} was quantitatively similar in glands from knockout mice, indicating that basic upstream signaling, as well as downstream exocytotic machinery remained intact, and the only detectable defect was in the Ca^{2+} influx mechanism. Gwack *et al.* reported that Orai1 knockout mice showed signs of eyelid irritation [79], and in the study by Xing *et al.* many, but not all mice, showed signs of inflammation in the eyes [57] (Figure 4). Since the mice are immune compromised, it is not

possible to determine if this is a primary result of impaired lacrimal secretion, lack of immune function, or a combination of both. However, since many mice showed no such symptoms, yet all mice tested exhibited loss of SOCE, it is clear that the SOCE phenotype is not secondary to this inflammation. It is interesting that defects in SOCE can affect exocrine function in two important ways, by triggering a pathological autoimmunity [80], or by failure of signaling for protein and fluid secretion [57].

Summary

Studies on Ca^{2+} signaling in lacrimal glands have provided important clues for our understanding of basic signaling mechanisms, especially with regard to store-operated Ca^{2+} entry mechanisms. In addition, these mechanistic studies provide possible insights to the causes and possible treatments of debilitating dry eye diseases.

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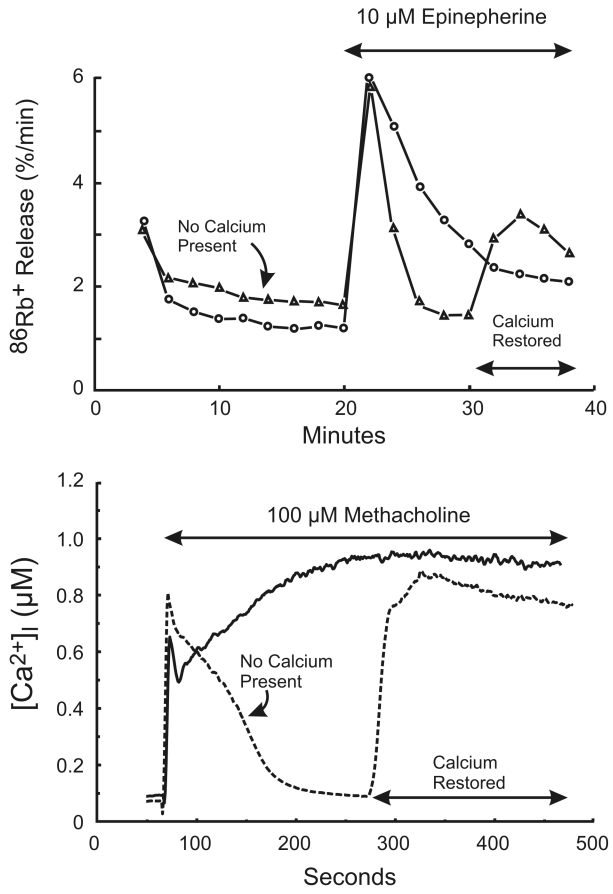

Figure 1. Two phases of Ca^{2+} signaling in in vitro lacrimal gland preparations

Top: Changes in intracellular Ca^{2+} in slices of rat lacrimal gland are inferred from the efflux rate of $^{86}Rb^+$. Redrawn from data originally presented in [11]. Bottom: Changes in intracellular Ca^{2+} in mouse lacrimal acinar cells are measured with the Ca^{2+} indicator, Fura-2. In both cases, in the absence of extracellular Ca^{2+}, the response is transient, and subsequently restored by addition of Ca^{2+}.

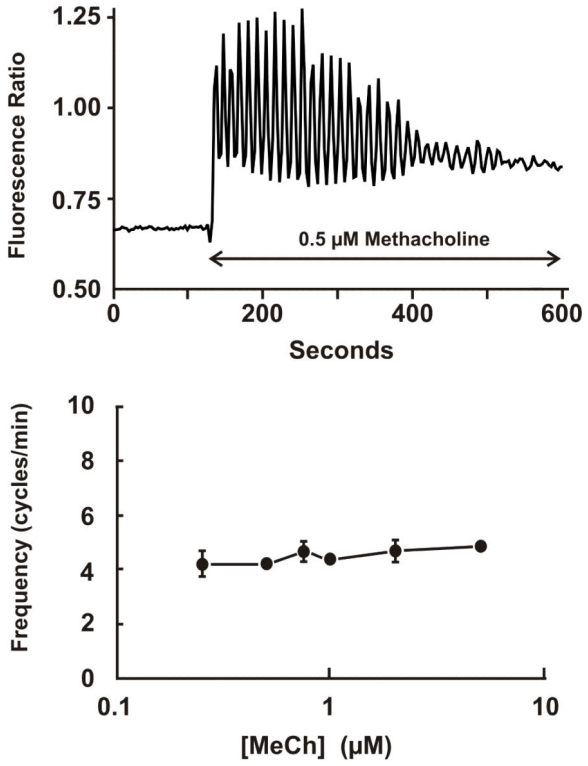

Figure 2. Muscarinic receptor-induced sinusoidal Ca^{2+} oscillations in mouse lacrimal cells

Top: A single, fura-2-loaded mouse lacrimal acinar cell was exposed to 0.5 μM methacholine (MeCh) inducing sinusoidal Ca^{2+} oscillations on an elevated basal level of Ca^{2+}. MeCh-induced oscillations occur over a narrow concentration range, and with frequencies that are relatively constant at different agonist concentrations (Bottom figure). These findings were originally published in [46].

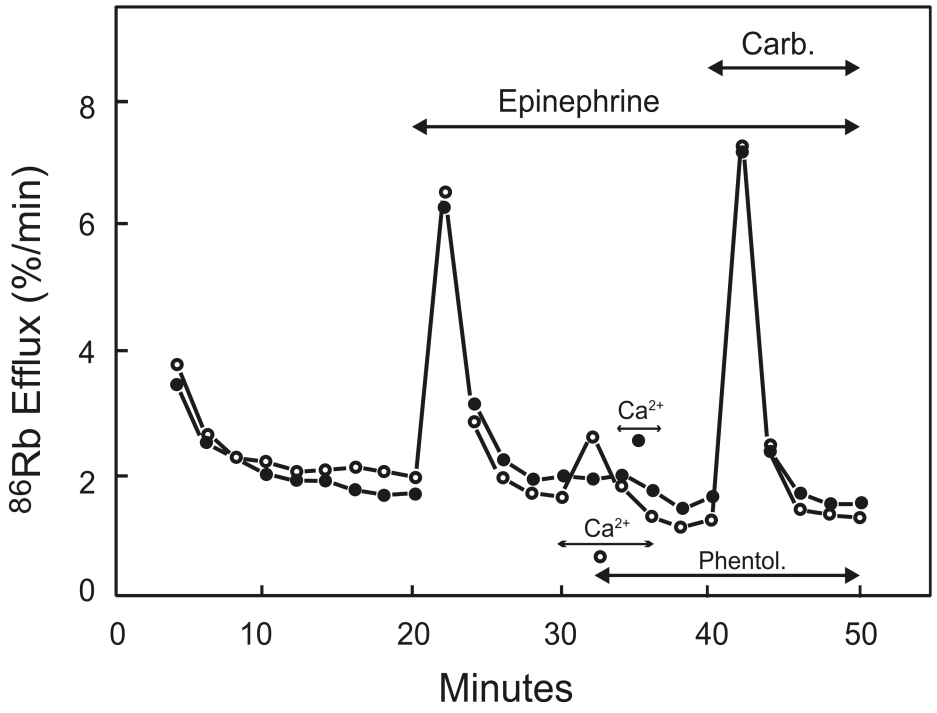

Figure 3. Evidence for the independence of Ca^{2+} influx from receptor activation

In both experiments, Ca^{2+} stores were discharged by addition of epinephrine in the absence of extracellular Ca^{2+}, the α-adrenergic receptors were then blocked by phentolamine (Phentol.), and the status of Ca^{2+} stores assessed by addition of carbachol (Carb.). In the experiment with open circles, Ca^{2+} was added before phentolamine, so Ca^{2+} could flow into the cell through presumed receptor activated channels. In the experiment with closed circles, phentolamine was added before Ca^{2+} such that receptor-activated channels would presumably be closed. Nonetheless, the stores were refilled with similar efficiency in both cases, indicating that it is not receptor activation *per se* that is responsible for Ca^{2+} entry and refilling of intracellular stores. Redrawn from data originally presented in [25].

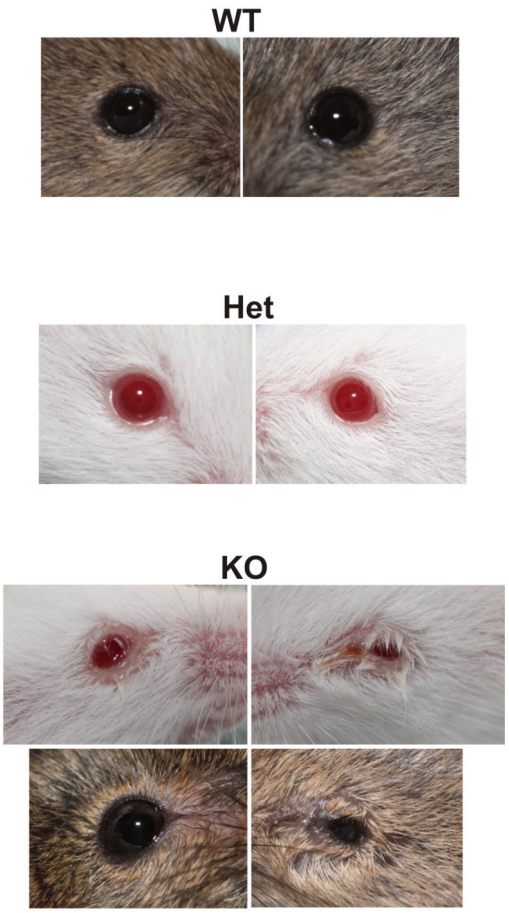

Figure 4. Appearance of eyes from wild type (WT), heterozygous (Het) and homozygous Orai1 knockout (KO) mice

The eyes from wild type and heterozygous mice appeared normal, while in many (but not all) cases the eyes from knockout animals showed signs of inflammation.