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Neural Regulation of Lacrimal Gland Secretory Processes: Relevance in Dry Eye Diseases

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Abstract

The lacrimal gland is the major contributor to the aqueous layer of the tear film which consists of water, electrolytes and proteins. The amount and composition of this layer is critical for the health, maintenance, and protection of the cells of the cornea and conjunctiva (the ocular surface). Small changes in the concentration of tear electrolytes have been correlated with dry eye syndrome. While the mechanisms of secretion of water, electrolytes and proteins from the lacrimal gland differ, all three are under tight neural control. This allows for a rapid response to meet the needs of the cells of the ocular surface in response to environmental conditions. The neural response consists of the activation of the afferent sensory nerves in the cornea and conjunctiva to stimulate efferent parasympathetic and sympathetic nerves that innervate the lacrimal gland. Neurotransmitters are released from the stimulated parasympathetic and sympathetic nerves that cause secretion of water, electrolytes, and proteins from the lacrimal gland and onto the ocular surface. This review focuses on the neural regulation of lacrimal gland secretion under normal and dry eye conditions.

Keywords

Sensory reflex; Efferent nerves; Afferent nerves; Neurotransmitters; Growth factors; Secretion; Signaling pathways

1. Introduction

The lacrimal gland, a tubuloacinar exocrine gland, secretes electrolytes, water, proteins, and mucins known as lacrimal gland fluid, into the tear film. The appropriate amount and composition of lacrimal gland fluid is critical for a healthy, intact ocular surface. As a small change in the concentration of tear electrolytes is correlated with dry eye syndrome, secretion of lacrimal gland electrolytes must be tightly regulated. Furthermore the lacrimal gland synthesizes and secretes a plethora of proteins with a variety of functions that help to nourish and protect the corneal and conjunctival epithelia and to regulate the function of these tissues. Protein secretion, similarly to electrolyte and water secretion, is highly regulated. In the context of the need for control of lacrimal gland fluid secretion, neural

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regulation plays an integral role controlling lacrimal gland protein, electrolyte, and water secretion and hence tears volume and composition. A rapid neural response is critical to meet the needs of the ocular surface both in protection from the stresses of the environment (temperature, humidity, mechanical, chemical, or pathogenic) and the requirements of the surface epithelia (growth control, wound healing, electrolyte transport, maintenance of the tear/aqueous humor barrier, and shedding of surface proteins).

The neural response that regulates lacrimal gland fluid secretion is an integral part of the lacrimal gland functional unit that consists of sensory afferent nerves of the cornea and conjunctiva, the efferent parasympathetic and sympathetic nerves that innervate the lacrimal gland, the lacrimal gland secretory cells, and the lacrimal gland excretory ducts (Figure 1). Stimulation of the plentiful afferent, sensory corneal and conjunctival nerves activates the efferent nerves to the lacrimal gland stimulating secretion of lacrimal gland electrolytes, water, and proteins that exit the gland as lacrimal gland fluid via the excretory ducts onto the surface of the eye. This is not a simple reflex, but rather the sensory input is processed in the lacrimal nucleus of the brain, which also processes input from other centers (e.g. emotional input) to generate a graded output. The stimulation is graded so that low levels of sensory nerve stimulation produce enough lacrimal gland fluid to cover the ocular surface as the pre-corneal tear film. More intense stimulation causes increased lacrimal gland fluid secretion to wash away deleterious compounds on the ocular surface and produce overflow tears.

Sensory nerves of the ocular surface also regulate secretion of the conjunctival goblet and stratified squamous cells and the corneal epithelial cells that contribute mucins, proteins, electrolytes, and water to the tear film. In turn these secretions can affect function of the sensory nerves. The neural regulation of corneal and conjunctival secretion has been recently reviewed (Lucarelli, Dartt et al. 2002; Dartt 2004; Burkett, Hodges et al. 2006).

This review will focus on neural stimulation of lacrimal gland secretion and will be divided into regulation of: 1) afferent sensory nerve stimulation from the ocular surface, 2) efferent parasympathetic and sympathetic activation of lacrimal gland secretory cells, and 3) the mechanism of protein, electrolyte, and water secretion from the lacrimal gland cells.

2. Structure of Lacrimal Gland

The main lacrimal gland is an almond-shaped gland that in many species, including humans and rabbits, is located within the bony orbit of the eye. In rats and mice, the main lacrimal gland is exorbital, lying just below the ear with its long axis perpendicular to the zygomatic arch, and is connected to the ocular surface with a single, long excretory duct (Venable and Grafflin 1940). The excretory duct of the exorbital lacrimal gland runs forward across the temporal muscle directly to the outer part of the upper eyelid. Just before reaching the lid, the duct is joined by the duct of the infraorbital gland. The lacrimal gland is tubuloacinar (also known as tubuloalveolar). In the cat, rabbit, and pig the gland is serous. In goat it is mucous and in the human, rat and dog it is mixed seromucous (Prince 1977). The main secretory cell in all lacrimal glands is the acinar cell that comprises about 80% of the gland (Figure 2). Acinar cells are pyramidal shaped cells that are linked together by apical tight junctions to form a ball in racemous glands and long tubules in tubuloacinar glands. The tight junctions separate the apical from the basolateral plasma membranes and are responsible for the polarization of the acinar cell and its ability to secrete electrolytes, water, and proteins into the lumen. The basolateral membrane contains the receptors for neurotransmitters, neuropeptides, and growth factors that initiate the secretory process. This membrane also contains ion transport proteins and ion channels necessary for initiating electrolyte and water secretion (Figure 2). A different set of ion transport proteins and ion channels are located in the apical membrane. The apical membrane is also the site of fusion

of the secretory granules that deliver lacrimal gland proteins into the lumen. The secretory proteins are synthesized in the acinar cells in the well-developed endoplasmic reticulum and Golgi apparatus that fill the basal portion of the cell. Most of the synthesized and enzymatically modified secretory proteins are stored in the secretory granules that pack the apical portion of the cell. Some secretory proteins are present in a different set of intracellular vesicles or are present as membrane bound proteins whose extracellular domain is shed. The different mechanisms of secretion and the proteins secreted by these mechanisms are discussed in Section IX.

The lumens of the acinar cells converge to form the excretory ducts that are lined by 1-2 layers of cuboidal duct cells. Although salivary glands contain multiple types of duct cells, the lacrimal gland contains few types of ducts especially compared to the submandibular salivary gland. The ducts are interlobular and intralobular and perhaps inter- and intra-lobular. Duct cells comprise about 10-12% of the gland. Similar to acinar cells, duct cells are joined on the luminal (apical) side by tight junctions creating polarized cells that contribute to the unidirectional secretion of lacrimal gland fluid. A major function of duct cells is to modify the primary lacrimal gland fluid from the acinar cells by secreting electrolytes and water. Mircheff estimated that lacrimal gland duct cells secrete about 30% of the lacrimal gland fluid (Mircheff 1983). Duct cells contain a slightly different arrangement of ion transport proteins and ion channels than acinar cells. Similar to acinar cells, duct cells also secrete proteins that they synthesize in endoplasmic reticulum and Golgi apparatus and store in apically located secretory granules or in the plasma membrane. However, there are substantially fewer granules in duct cells than in acinar cells.

The third major cell type present in the lacrimal gland are the myoepithelial cells. These cells surround the basal side of both acinar and duct cells (Figure 3). They are flat stellate cells that form a discontinuous network subjacent to the nerves and blood vessels and interfacing between the acinar and duct cells and the extracellular space. Myoepithelial cells are only present in the lacrimal, salivary, and mammary glands. These cells are identified by the presence of α -smooth muscle actin on their stress fibers. In the mammary gland myoepithelial cells are well-known to contract stimulated by the hormone oxytocin to release pre-formed secretory product (milk) from the filled duct system. In the salivary glands there is indirect evidence that myoepithelial cells contract at the initiation of secretion. In the guinea pig lacrimal gland myoepithelial cells have been observed to contract in response to the cholinergic agonist carbachol, but not to the adrenergic agonists epinephrine and norepinephrine (Sato, Sano et al. 1997). In fact myoepithelial cells contain multiple G protein-coupled receptors and other signaling components, but the function of these cells in the production of lacrimal gland fluid is unknown.

The lacrimal gland also contains a small but important population of lymphocytes, plasma cells, mast cells (in rodents), dendritic cells, and macrophages. The plasma cells express immunoglobulins, especially dimeric IgA that along with J chain and secretory component forms secretory IgA, an important component of the secretory immune system that helps to protect the surface of the eye. An upregulation of mast cells and lymphocytes plays a role in lacrimal gland pathology (Williams, Singh et al. 1994; Zoukhri, Hodges et al. 1998; Rios, Horikawa et al. 2005).

3. Regulation of Sensory Reflexes from the Ocular Surface

3.1. Afferent Sensory Regulation

The first segment of the lacrimal gland functional unit that regulates lacrimal gland secretion is the activation of sensory nerves in the corneal and conjunctival epithelia. The ocular surface epithelia are richly endowed with sensory nerve endings that respond to changes in

the environment causing a rapid secretion of lacrimal gland fluid to wash away and chemically neutralize foreign substances that have entered the tear film (Mutch 1944; Ruskell 1971; Ruskell 2004). Stimulation of corneal sensory nerves causes both fluid secretion from and vasodilation in the lacrimal gland. Lacrimal gland fluid secretion is dependent upon vasodilation with increased blood flow augmenting secretion and decreasing blood flow inhibiting stimulated-secretion (Botelho, Martinez et al. 1976). In animals that were cervically sympathectomized, vasodilation and tear volume (an indirect measurement of lacrimal gland secretion) responses were blocked by prior treatment with hexamethonium that blocks the autonomic ganglia and by a local anesthetic injected into the ganglion (Yasui, Karita et al. 1997). These findings suggest that stimulation of corneal sensory nerves stimulate the lacrimal gland by a trigeminal-parasympathetic reflex (Yasui, Karita et al. 1997). This reflex was mediated by the facial nerve, but not glossopharyngeal nerve. Activation of the facial nerve stimulated lacrimal gland blood flow and increased tear volume (an indirect measurement of lacrimal gland secretion) with similar intensity and duration of stimulation, but the efferent mediators of the two functions differed substantially. Secretion was blocked by muscarinic antagonists, but vasodilation was not blocked by muscarinic, VIP, or sympathetic antagonists. The mechanism responsible for the coupling between lacrimal gland vasodilation and fluid secretion is unknown.

Sensory corneal and conjunctival nerves are of the thin myelinated A-delta type or unmyelinated C type. They lose their myelin sheath when they enter the stroma a branch extensively to form a subepithelial plexus (Belmonte, Acosta et al. 2004). This plexus branches into thin nerves that ascend into the basal layer of the epithelium where they course parallel to the surface forming leash type of nerve endings that terminate in the apical layers of the epithelium. Thus the nerve endings are exceedingly close to the surface and can easily react to external changes in the tear film. Morphologically the ocular surface sensory nerves appear homogeneous with almost 60% containing the neuropeptide CGRP in their nerve endings and about 20% containing substance P (Muller, Marfurt et al. 2003). Electrophysiologically the nerves are very different and can be divided into mechanoreceptor (low threshold), mechano-nociceptor (high threshold), polymodal nociceptor, and cold receptor (Acosta, Tan et al. 2001) (Figure 4). About 20% of the sensory nerves are mechanoreceptor and mechano-nociceptor and respond to mechanical forces of the magnitude that can damage the cornea. These nerves are responsible for the acute pain sensation that occurs with mechanical contact with the cornea. About 70% of the nerves are polymodal nociceptors that are stimulated by mechanical forces, chemical irritants, and endogenous chemical mediators released by damaged tissue, inflammatory cells, or plasma extravasated from the blood vessels (Belmonte, Acosta et al. 2004). The polymodal nociceptors cause the sharp pain of mechanical stimulation as well as the irritative pain caused by chemical irritation, heat, cold, or inflammation. Finally 10-15% of the nerve fibers are cold sensitive. Thus the external types of stimuli, mechanical, chemical, or temperature, activate corneal and conjunctival sensory nerves.

Activation of the sensory nerves to conduct in the dromic direction, that is toward the brain causes lacrimal gland secretion. The polymodal nociceptors in the cornea are the primary nerves that stimulate reflex tear secretion and hence lacrimal gland fluid secretion (Acosta, Peral et al. 2004). Activation of corneal mechanoreceptors and cold receptors was less effective in stimulating tear secretion. In contrast, stimulation of conjunctival sensory receptors did not cause tear secretion.

Sensory nerves can also be activated to conduct impulses in the antidromic direction, which releases the sensory neuropeptides into the corneal and conjunctival epithelia (Belmonte, Acosta et al. 2004). This antidromic stimulation can be the result of tissue injury or inflammation, which release inflammatory mediators that change the electrical activity of

the nerves causing release of neurotransmitters. The release CGRP and Substance P produce neurogenic inflammation consisting of vasodilation, plasma extravasation, and cytokine release. The excited nerve endings propagate electrical impulses centripetally and can stimulate non-injured branches of the parent axon to release neuropeptides without being injured.

Denervation of the sensory nerves has a profound effect on lacrimal gland function. Meneray et al (Meneray, Bennett et al. 1998) ablated the ophthalmic division of the trigeminal ganglion in the rabbit that depleted the sensory nerves of both the cornea and the lacrimal gland itself. In the denervated eye, lacrimal gland acinar cells demonstrated a massive accumulation of secretory granules typical of denervated secretory glands, as stimulation of vesicle release had been prevented. When exogenous cholinergic or β -adrenergic agonists were added to denervated glands in vitro there was an increase in protein secretion compared to non-denervated glands. This is the classical denervation supersensitivity response. Interestingly when tissues were stimulated with phorbol esters that activate the classical and novel, but not atypical, forms of protein kinase C (PKC), or with forskolin that activates adenylate cyclase, protein secretion was not increased. PKC and adenylate cyclase are steps in the signaling pathways activated by cholinergic and β -adrenergic agonists, respectively. For the cholinergic signaling pathway, this finding suggests the following possible effects of denervation that would cause increased secretion by denervation supersensitivity: 1. alteration of a different, non-PKC-dependent pathway activated by this agonist, such as the Ca^{2+} - or phospholipase D-dependent pathways, 2. activation of steps in the pathway distal to activation of PKC isoform, or 3. targeting of only one or two PKC isoforms. Phorbol esters activate multiple PKC isoforms that can have opposing functional roles. For β -adrenergic agonists, the finding suggests that denervation alters this pathway distal to adenylate cyclase, that is the receptor itself is affected or its coupling to adenylate cyclase is altered. Another possibility is that β -adrenergic agonists stimulate a signaling pathway in addition to the adenylate cyclase-dependent one. As VIP is an additional stimulus of lacrimal gland secretion, sensory denervation could also have affected the response to VIP as occurs in the parotid gland (McMillian and Talamo 1989). In this case VIP could be depleted from the parasympathetic nerves and VIP binding to its receptor increased.

Results from the Meneray et al study (Meneray, Bennett et al. 1998) suggest that activation of sensory nerves from the cornea and conjunctiva play a critical role in stimulation of lacrimal gland secretion. As denervation of the ophthalmic division of the trigeminal ganglion also blocks activation of the lacrimal gland sensory nerves, loss of these nerves could also have contributed to the denervation. As the sensory innervation of the lacrimal gland is minimal compared to the sensory innervation of the cornea, denervation alters both the parasympathetic and sympathetic innervation that is most abundant in the gland. Therefore, it is most likely that it is the sensory innervation of the cornea is responsible for the vast majority of the effect of denervation obtained in the Meneray et al (Meneray, Bennett et al. 1998) study and is the neural driving force for lacrimal gland secretion.

3.2. Efferent Parasympathetic and Sympathetic Regulation

Although the lacrimal gland is innervated by both the parasympathetic and sympathetic nerves, the parasympathetic system predominates, both anatomically and functionally (Figure 5). Immunofluorescence microscopy with antibodies to the neurotransmitters and neuropeptides, or the enzymes that synthesize these compounds, demonstrate that in all species there is a dense innervation of the lacrimal gland by parasympathetic nerves (Ruskell 1969; Ruskell 1971; Dartt, Baker et al. 1984). Use of anti-vesicular acetylcholine transporter (Ding, Walcott et al. 2001; Rios, Horikawa et al. 2005) demonstrates a plentiful parasympathetic innervation in the C57B6 and BalbC mouse lacrimal gland. There is

controversy over the density of the sympathetic innervation. As summarized by Ding et al (Ding, Walcott et al. 2003) the extent of sympathetic innervation is not only species dependent, but also the object of discrepancy in the literature. For rat, mouse, guinea pig, and monkey lacrimal gland, some reports show dense and some show sparse sympathetic innervation. Ding et al (Ding, Walcott et al. 2003) found different densities of sympathetic innervation in distinct areas of the gland. Careful re-evaluation of the density of sympathetic nerves with multiple markers of sympathetic nerves including tyrosine hydroxylase, dopamine beta hydroxylase, and phenylethanolamine-N-methyltransferase is warranted. In spite of the discrepancy concerning the gland, α_1 -adrenergic agonists consistently stimulated lacrimal gland protein secretion (Bromberg and Welch 1985; Bromberg, Cripps et al. 1986; Dartt, Rose et al. 1994; Ding, Walcott et al. 2003) and activate ion channels (Parod and Putney 1978; Parod, Dambach et al. 1980).

In contrast to the discrepancy in the density of sympathetic innervation, functional studies consistently indicate that a loss of either preganglionic (facial nerve) or postganglionic parasympathetic, but not sympathetic, innervation causes a precipitous drop in lacrimal gland secretion. Sympathetic denervation of the rabbit lacrimal gland by ablating the superior cervical ganglion did not alter lacrimal gland acinar morphology and did not induce denervation supersensitivity of protein secretion (Meneray, Bennett et al. 1998). In contrast there is overwhelming evidence that loss of parasympathetic innervation blocks lacrimal gland function. The parasympathetic pathway to the lacrimal gland begins in the superior salivary nucleus in a cranial nerve nucleus in the pontine tegmentum and courses through the geniculate ganglion without synapsing and then emerges as the greater superficial petrosal nerve, which joins the deep petrosal nerve to form the vidian nerve. The vidian nerve terminates in the pterygopalatine ganglion. These nerves form the preganglionic parasympathetic nerve supply the lacrimal gland. The postganglionic axons are the output of the pterygopalatine ganglion project to the lacrimal gland and supply the parasympathetic innervation of the lacrimal gland (Ruskell 1970; Goadsby 1990; Ten Tusscher, Klooster et al. 1990; van der Werf, Baljet et al. 1996; Yasui, Karita et al. 1997; Hoshino, Yoshizaki et al. 1998; Toth, Boldogkoi et al. 1999).

According to multiple studies, both preganglionic and postganglionic denervation alters acinar cell morphology and blocks lacrimal gland function (Ruskell 1969; Butler, Ruskell et al. 1984; Toda, Ayajiki et al. 2000; Toshida, Nguyen et al. 2007). In these studies lacrimal gland function was evaluated by measuring tear volume or flow, an indirect measurement of lacrimal gland function. Another measure of lacrimal gland function used was a microarray analysis of genes in lacrimal glands with and without preganglionic denervation. In the denervated glands there was a downregulation of genes for the Golgi apparatus and the endoplasmic reticulum, but an upregulation of genes for cytoskeletal and extracellular matrix components, inflammation, and apoptosis (Nguyen, Toshida et al. 2004; Nguyen, Vadlamudi et al. 2006). Furthermore, postganglionic denervation increased the concentration of three tear proteins one of which was identified as transferrin, in the lacrimal gland (Salvatore, Pedroza et al. 1999). In a later study, in denervated animals, tear protein concentration was decreased consistent with decreased lacrimal gland secretion. Conversely, the concentration of transferrin and two other unidentified proteins was increased in tears, whereas a decrease was expected. One explanation is that other tissues, in addition to the lacrimal gland, secrete these proteins and these tissues were not affected by the denervation. This explanation would then imply that measurement of tear proteins, unlike tear volume, is not an accurate measurement of lacrimal gland function.

Most studies denervate the lacrimal gland by cutting the lacrimal nerves. Preganglionic denervation by sectioning the greater superficial petrosal nerve is more specific to the parasympathetic innervation as disruption of the lacrimal nerve and the pterygopalatine

ganglion also affects minor populations of sympathetic and sensory fibers (Toshida, Nguyen et al. 2007). Sectioning of the greater superficial petrosal nerve in rabbits caused a profound dry eye characterized by a 70% decrease in tear production and an accumulation of secretory granules in the denervated gland. This study provides direct evidence that parasympathetic nerves are the primary regulatory of lacrimal gland secretion.

Another model of parasympathetic denervation are the neurturin or GFR α -2 knockout mice (Heuckeroth, Enomoto et al. 1999; Rossi, Luukko et al. 1999). Neurturin is a member of the transforming growth factor- β family of growth factors that serve as neurotrophic factors for neurons. The receptor for neurturin is GFR α -2 that functions by activating the non-receptor tyrosine kinase c-Ret. Neurturin is essential for the development and maintenance of selective postganglionic parasympathetic neurons including those that innervate the lacrimal gland, salivary gland, and small intestine. Neurturin is also essential for trigeminal nerve development and maintenance. Both neurturin and GFR α -2 knockout mice are deficient in the same postganglionic parasympathetic neurons (check), although there are subtle differences between the two types of mice. Neurturin deficient mice have dry eye (Song, Li et al. 2003). Relevant to the lacrimal gland, these mice have decreased tear production and decreased corneal sensitivity compared to control mice, suggesting defects in both the afferent sensory and the efferent parasympathetic neural drive of lacrimal gland secretion.

For all models of afferent and efferent neural regulation of lacrimal gland secretion, additional research is necessary to measure lacrimal gland secretion, both protein as well as electrolyte and water, using methods that directly evaluate secretion by this gland as all studies on denervation measured tear rather than lacrimal gland fluid secretion. For in vivo studies cannulation of the lacrimal gland excretory duct to measure the volume or rate of secretion along with collection and analysis of the fluid would be recommended. For in vitro studies measurement of specific lacrimal gland secretory proteins rather than total protein would be suggested. This is of particular importance as recent studies have shown that neurotransmitter agonists can cause release of membrane-spanning proteins, such as EGF and secretory component, by ectodomain shedding (Chen, Hodges et al. 2006; Evans, Zhang et al. 2008). Other lines of investigation could include determining the cellular signaling mechanisms by which denervation supersensitivity alters lacrimal gland secretion.

4. Overview of Neural Stimulation of Lacrimal Gland Secretion

Activation of either parasympathetic or sympathetic nerves releases neurotransmitters that control lacrimal gland secretion of both proteins and electrolytes and water (Figure 6). The major neurotransmitters that regulate secretion are the parasympathetic neurotransmitters acetylcholine and VIP, as well as the sympathetic neurotransmitter norepinephrine. These agonists are all stimulatory and each activates a different, separate signaling pathway, although these pathways interact. The signaling pathways activated by acetylcholine, VIP, and norepinephrine, as well as their interaction, will be discussed in detail concentrating on the literature since the last comprehensive review (Hodges and Dartt 2003). Sympathetic nerves also release the stimulatory neurotransmitters neuropeptide Y. Furthermore, the enkephalins provide an inhibitory pathway that blocks lacrimal gland secretion. These two agonists will not be included in the present review as there is no additional literature on the role of these compounds in lacrimal secretion since 2003.

5. Neural Stimulation of Lacrimal Gland Secretion- Cholinergic, Parasympathetic Pathway

5.1. Cholinergic Agonist Effects on Secretion

When considering the target tissue of the nerves, the lacrimal gland, the evidence is overwhelming that cholinergic agonists stimulate lacrimal gland protein and fluid (electrolyte and water) secretion. Evidence is based on drugs effects in humans, in vivo studies in rabbits and rats using denervation or systemic administration of agonists and antagonists, and in vitro studies in rabbits, rats, and mice using tissue pieces or collagenase-digested acini (groups of acinar cells). In humans, anticholinergic drugs, for example tolterodine, atropine, oxybutyrin, tiotropium, and hyoscine butylbromide or drugs with anticholinergic side effects, such as tricyclic antidepressants and antihistamines, are known to cause dry eye, which is indirect evidence for cholinergic stimulation of the lacrimal gland. In addition to denervation methodology described in the preceding sections, the effect of muscarinic antagonists suggests a major role for parasympathetic nerves in stimulation of lacrimal gland secretion as systemic administration of the muscarinic cholinergic antagonist scopolamine produces dry eye in mice that is exacerbated by a desiccating environment (Dursun, Wang et al. 2002). Intraarterial or intraperitoneal systemic administration of acetylcholine, pilocarpine, or other cholinergic agonists stimulates lacrimal gland protein and fluid secretion measured from the cannulated lacrimal gland excretory duct of anesthetized rabbits (Dartt and Botelho 1979; Dartt, Knox et al. 1980; Dartt, Moller et al. 1981; Ubels, Foley et al. 1986; Rismondo and Ubels 1987; Rismondo, Ubels et al. 1988; Ubels, Rismondo et al. 1989). Incubation of lacrimal gland pieces or acini with the cholinergic agonist carbachol stimulates protein secretion measured by peroxidase secretion from rat and mouse (Herzog, Sies et al. 1976; Dartt, Baker et al. 1984; Dartt, Donowitz et al. 1984), and β -hexosaminidase secretion from rabbit (Andersson Exp Eye Res 1081-1088-2006).

5.2. Stimulatory Muscarinic Cellular Signaling Pathways

5.2.1. Introduction—The cellular signaling pathways activated by cholinergic agonists have best been studied in vitro and thus can only be directly connected to protein, but not electrolyte and water, secretion. Two recent developments one on the isolation of lacrimal gland ducts (Ubels, Hoffman et al. 2006) and another on the construction of an artificial lacrimal gland have provided needed methodology for studying electrolyte and water secretion in vitro (Ubels, Hoffman et al. 2006; Selvam, Thomas et al. 2007; Selvam, Thomas et al. 2007). These will be discussed in Section X. The individual components of the cholinergic signaling pathway discussed here have only been connected to protein secretion, however, these components are likely to be connected to electrolyte and water secretion as well.

5.2.2. Inositol trisphosphate and Ca^{2+} Signaling—The cholinergic receptors on lacrimal gland cells are the muscarinic M_3 (glandular) subtype (M_3AChR) (Hootman and Ernst 1981; Hootman, Picado-Leonard et al. 1985; Mauduit, Jammes et al. 1993) (Figure 7). The M_3AChR is coupled to the $\text{G}_{q/11\alpha}$ subtype of G protein (Meneray, Fields et al. 1997) that is then connected to activation of the enzyme phospholipase $\text{C}\beta$ that breaks down the membrane phospholipids phosphatidylinositol bisphosphate into 1,4,5-inositol trisphosphate (InsP_3) and diacylglycerol (DAG) (Godfrey and Putney 1984; Morris, Gallacher et al. 1987; Dartt, Dicker et al. 1990; Mauduit, Jammes et al. 1993). InsP_3 is water soluble and binds to its receptor on the endoplasmic reticulum. There are 3 distinct subtypes of InsP_3 receptors types 1-3 that are expressed to varying degrees on in different cell subtypes (Wojcikiewicz 1995). Activation of these receptor subtypes in non-excitable cells such as the lacrimal gland

results in a complex array of temporal and spatial intracellular $[Ca^{2+}]$ signals that are initiated in defined regions. In lacrimal gland acini, as in pancreatic and salivary gland acini, the Ca^{2+} signal is initiated from the apical area of the acinar cell close to the secretory granules, before the basal area, the location of the neurotransmitter receptors and the ion transport pumps and channels thought to initiate fluid secretion (Tan, Marty et al. 1992; Toescu, Lawrie et al. 1992; Gromada, Jorgensen et al. 1993; Satoh, Sano et al. 1997).

InsP₃ interaction with its receptor causes a rapid, immediate release of intracellular Ca^{2+} represented by a spike in Ca^{2+} response that quickly peaks. The subsequent response can be waves of Ca^{2+} propagated across cells or Ca^{2+} oscillations within cells whose shape controls the functional response (Yule 2001; Yule, Straub et al. 2003). The shape of the Ca^{2+} response is dependent upon the type of InsP₃ receptor present and the cellular localization of the receptor subtypes. The receptor subtypes are differentially sensitive to InsP₃ and Ca^{2+} , the major regulators of the InsP₃ receptor (Yule, Straub et al. 2003). The InsP₃ receptor subtypes present in lacrimal gland acini are unknown, but in the parotid the majority of receptors are type 2, with a significant complement of type 3 and about 5% type 1 (Zhang, Wen et al. 1999; Giovannucci, Bruce et al. 2002). In pancreatic acini, all three subtypes of receptors were present in the luminal area and thus could account for the initial release of Ca^{2+} from the apical pole (Yule, Ernst et al. 1997). Studies by Mak et al implicate the type 3 receptor in the initial Ca^{2+} release at the luminal membrane in exocrine cells (Mak, McBride et al. 2001; Mak, McBride et al. 2001).

After InsP₃ binds to its receptor to release intracellular Ca^{2+} in lacrimal gland acini, as well as in most cells in general, Ca^{2+} influx occurs across the basolateral membrane causing the plateau phase of the Ca^{2+} response (Dartt, Dicker et al. 1990; Bird, Rossier et al. 1991). The depletion of the intracellular Ca^{2+} stores signals the plasma membrane to increase Ca^{2+} influx to reload the intracellular stores (endoplasmic reticulum) (Figure 8). This process was termed capacitative or store operated calcium entry and was discovered by Putney (Putney 1986) using lacrimal and parotid acini. An excellent review by Putney of the history of capacitative Ca^{2+} entry can be found in *Cell Calcium* (Putney 2007).

Use of thapsigargin which blocks Ca^{2+} reuptake into the endoplasmic reticulum by Ca^{2+} ATPase provided a tool to measure capacitative Ca^{2+} influx. In lacrimal gland acini use of thapsigargin in the absence of extracellular Ca^{2+} depletes the intracellular Ca^{2+} stores, as Ca^{2+} continuously leaks out of the endoplasmic reticulum. The store usually refills due to the activity of the Ca^{2+} ATPase that pumps Ca^{2+} back into the stores, but thapsigargin prevents this. When extracellular Ca^{2+} is reintroduced, Ca^{2+} influx occurs that represents capacitative Ca^{2+} entry (Putney 1990; Zoukhri, Hodges et al. 2000). Importantly, the activation of Ca^{2+} influx by thapsigargin is independent of activation of phospholipase C. The Ca^{2+} entry pathway has been identified electrophysiologically in mast cells by Hoth and Penner (Hoth and Penner 1992) and termed calcium-release-activated Ca^{2+} current (I_{CRAC}). Until recently neither the channel nor its mechanism of activation was identified.

Three fundamental mechanisms were proposed for transmitting the signal for the refilling of the Ca^{2+} stores from the plasma membrane to activate I_{CRAC} including use of a diffusible compound, vesicle secretion, and conformational coupling (Putney 2007). In support of the diffusible factor hypothesis, a diffusible factor termed calcium influx factor (CIF) has been isolated, but not yet identified (Randriamampita and Tsien 1993; Bolotina and Csutora 2005). Investigation of the vesicle secretion theory has not been continued.

For the conformational coupling theory, the hypothesis is that a fall in luminal Ca^{2+} in the endoplasmic reticulum would induce a conformational change in the InsP₃ receptor this would be transmitted directly to the plasma membrane by protein-protein interaction (Irvine

1990). For this interaction to occur the endoplasmic reticulum and plasma membrane store-operated Ca^{2+} channels need to be closely associated. Until recently there was limited direct evidence to support the conformational coupling theory. In 2005, Stim and in 2006 Orai proteins were discovered revolutionizing the field of capacitative Ca^{2+} entry via I_{CRAC} . Stim1 is a transmembrane protein with a single transmembrane segment. Stim1, but not Stim2, acts as a sensor of Ca^{2+} levels in the endoplasmic reticulum via an EF-hand domain that extends into the endoplasmic reticulum lumen (Putney 2007) (Figure 8). When the endoplasmic reticulum Ca^{2+} stores are depleted, Stim1 translocates into punctate structures adjacent to the plasma membrane (Liou, Kim et al. 2005). Orai is a plasma membrane protein that has four transmembrane domains (Feske, Gwack et al. 2006). Although Orai has no signaling or channel-like domains, Orai functions as a Ca^{2+} channel. Upon depletion of endoplasmic Ca^{2+} stores, Stim1 translocates into puncta near the plasma membrane where it can interact with Orai and induce Ca^{2+} influx at the sites of interaction (Luik, Wu et al. 2006). Stim1 and Orai have not yet been identified in the lacrimal gland, but are extremely likely to function as the store-operated Ca^{2+} channel in acini.

In the lacrimal gland cholinergic agonists stimulate protein secretion with an initial rapid increase that gradually decreases with time (Herzog, Sies et al. 1976; Putney, VandeWalle et al. 1978; Dartt, Baker et al. 1984; Andersson, Hamm-Alvarez et al. 2006). The initial rapid response is not dependent upon extracellular Ca^{2+} , but was dependent upon intracellular Ca^{2+} , consistent with this rapid phase being initiated by InsP_3 -dependent Ca^{2+} release (Hodges, Dicker et al. 1992). The slower phase is dependent upon extracellular Ca^{2+} consistent with the dependence of this phase of secretion on capacitative Ca^{2+} influx (Putney, VandeWalle et al. 1978; Mauduit, Herman et al. 1983; Dartt, Donowitz et al. 1984; Mauduit, Herman et al. 1984).

5.2.3. Protein Kinase C signaling—Activation of phospholipase C produces diacylglycerol in addition to InsP_3 . Diacylglycerol activates a family of enzymes known as protein kinase C (PKC), that contains 11 different isoforms divided into three groups, classical, novel, and atypical. PKC α , - β I, - β II, and - γ are classical (c) isoforms that are activated by Ca^{2+} and phospholipids. PKC- δ , - ϵ , - η , and - θ are novel (n) isoforms activated by phospholipids, but not by Ca^{2+} . PKC λ , and - ζ , are atypical (a) isoforms that not activated by either Ca^{2+} or phospholipids. The lacrimal gland contains cPKC α , nPKC δ , nPKC ϵ , and aPKC λ , (Zoukhri, Hodges et al. 1997). In general PKC isoforms have cell and tissue specific localizations and functions. In the lacrimal gland cPKC α was present on the acinar basolateral and luminal membranes, nPKC δ in acinar cell cytoplasm (diffuse cellular binding) and on myoepithelial cells, nPKC ϵ in the acinar cell cytoplasm (diffuse cellular binding), basolateral membranes, and apical membrane system that looked similar in appearance to the actin network, and aPKC λ , on an endomembrane system that could be by endoplasmic reticulum or Golgi apparatus (Zoukhri, Hodges et al. 1997). Using myristoylated PKC isoform-specific pseudosubstrate inhibitors, Zoukhri et al found that cholinergic agonists use cPKC α , nPKC δ , and nPKC ϵ to induce protein secretion (Zoukhri, Hodges et al. 1997). This is consistent with the ability of phorbol esters that activate cPKC and nPKC isoforms to stimulate protein secretion (Dartt, Ronco et al. 1988; Andersson, Hamm-Alvarez et al. 2006).

Even though cholinergic agonists use both Ca^{2+} and PKC to stimulate protein secretion, either an increase in intracellular $[\text{Ca}^{2+}]_i$ or activation of PKC induces protein secretion. The calcium ionophore, ionomycin, increased the intracellular $[\text{Ca}^{2+}]_i$ and evoked exocytosis as measured as an increase in membrane capacitance of single acinar cells (Sundermeier, Matthews et al. 2002). Similarly transfection of acinar cells with adenovirus that expressed constitutively active (myristoylated) cPKC α increased basal protein secretion without changing the intracellular $[\text{Ca}^{2+}]_i$ (Hodges, Raddassi et al. 2004). Furthermore,

constitutively active cPKC α inhibited cholinergic-agonist stimulated protein secretion. cPKC α is the major PKC isoform activated by cholinergic agonists to stimulate secretion and cholinergic agonists activate cPKC α to phosphorylate the appropriate substrates to cause secretion. Addition of constitutively active cPKC α did not further increase secretion. As exocytosis is well known as a Ca²⁺ dependent process and the rate of exocytosis is dependent on the third power of the [Ca²⁺]_i, it is not clear how activated cPKC α stimulates protein secretion without increasing the intracellular [Ca²⁺]_i, unless activated cPKC α increases a microdomain of Ca²⁺. Thus it appears that an increase in intracellular [Ca²⁺]_i alone or activation of cPKC α alone is necessary and sufficient to cause protein secretion.

In spite of the independence of the cholinergic agonist-stimulated Ca²⁺ and PKC pathways, these pathways can interact. Activation of PKC can alter the Ca²⁺ influx pathway as inhibition of PKC with the non-selective PKC inhibitor staurosporine decreased the plateau Ca²⁺ in response to cholinergic agonists and preactivation of cPKC and nPKC isoforms with phorbol esters decreased both the peak and plateau Ca²⁺ response to cholinergic agonists (Zoukhri, Hodges et al. 2000). This is in agreement with the work of Tan and Marty (Tan and Marty 1991), Berrie and Elliot (Berrie and Elliott 1994), and Gromada et al (Gromada, Jorgensen et al. 1995) that PKC isoforms decrease the production of InsP₃, as well as the Ca²⁺ influx pathway. Using the myristoylated pseudosubstrate inhibitors, Zoukhri et al found that nPKC δ and nPKC ϵ , but not cPKC α , inhibited Ca²⁺ influx (Zoukhri, Hodges et al. 2000).

5.3. Inhibitory Muscarinic Cholinergic Signaling Pathways

Surprisingly, cholinergic agonists not only activate pathways that stimulate secretion, they also activate pathways that attenuate secretion. Cholinergic agonists activate p44-p42 mitogen-activated protein kinase (MAPK), also known as extracellular regulated kinase (ERK) 1/2, that decreases cholinergic-agonist activated protein secretion (Ota, Zoukhri et al. 2003). This is based on the finding that the MAPK inhibitor U0126 further increases cholinergic agonist-stimulated secretion. Cholinergic agonists activate MAPK by increasing the increasing the intracellular [Ca²⁺]_i, and activating PKC, which then induced the activity of the non-receptor tyrosine kinases Pyk2 and Src (Hodges, Rios et al. 2006). The mechanism by which cholinergic agonists activate MAPK in the lacrimal gland was unexpected as cholinergic agonists usually transactivate the EGF receptor, attracting Grb2 and Shc that activate SOS to stimulate Ras activity to induce Raf, Mek, and then MAPK. Furthermore, activation of MAPK is usually stimulatory. This type of stimulatory cholinergic agonist, EGF receptor, MAPK pathway regulates conjunctival goblet cell secretion (Kanno, Horikawa et al. 2003).

In the lacrimal gland cholinergic agonists activate phospholipase D (PLD), in addition to stimulating phospholipase C activity (Zoukhri and Dartt 1995). PLD hydrolyzes phospholipids similarly to phospholipase C, but in contrast to phospholipase C, PLD prefers phosphatidylcholine instead of inositol phospholipids. Activation of PLD produces phosphatidic acid that can function as a signaling molecule or can be degraded to diacylglycerol. In unpublished data, Hodges et al (Hodges RR and Dartt DA Invest Ophthalmol Vis Sci 2007 48:E-abstract 1909) found that it was PLD1 that was activated by cholinergic agonists. Using the PLD inhibitor 1-butanol Hodges et al demonstrated that stimulation of PLD activity attenuates cholinergic agonist-induced protein secretion. Not surprisingly, PLD worked via MAPK.

What could be the function of the inhibitory pathways that have been found in the rat lacrimal gland? These inhibitory pathways could regulate the shape or magnitude of the secretory response. Cholinergic agonists cause a rapid increase in protein secretion that peaks before decelerating. Activation of the inhibitory pathways could put the brake on the

secretory process. The inhibitory pathways could also reduce the magnitude of the secretory response. As both parasympathetic and sympathetic nerves stimulate both protein and fluid secretion, there is no negative regulation of secretion except through delta opioid receptors activated by endogenous enkephalins (Cripps and Bennett 1992). The inhibitory pathways could provide an opposing effect on secretion in the absence of inhibitory agonists.

6. Neural Stimulation of Lacrimal Gland Secretion- VIP, Parasympathetic Pathway

Parasympathetic nerve release VIP in addition to the cholinergic agonist acetylcholine. VIP is an important regulator of tear production in humans (Gilbard, Dartt et al. 1988). This was illustrated by a patient who had a VIP secreting tumor, a VIPoma that raised the circulating VIP levels. This individual had an increased tear volume and decreased tear osmolarity compared to aged matched controls indicating that VIP increases tear production in humans and the effect was most likely by stimulating lacrimal gland fluid secretion.

VIP works by binding to its receptors VIPAC1, which is on the basolateral membrane of acinar cells and VIPAC2, which is on myoepithelial cells (Hodges, Zoukhri et al. 1997) (Figure 9). The VIP receptor interaction activates the G protein $G_s\alpha$ that stimulates adenylyl cyclase (Hodges, Zoukhri et al. 1997; Meneray, Fields et al. 1997). Adenylyl cyclase increases intracellular cAMP level, which in turn activates protein kinase A (PKA). PKA stimulates secretion by phosphorylating unidentified target proteins. Using a myristoylated PKA peptide inhibitor (PKI) based on the pseudosubstrate of PKA, VIP stimulated protein secretion was shown to be inhibited by about 70% (Hodges, Zoukhri et al. 1997) suggesting that most, but not all, of VIP-stimulated protein secretion is cAMP dependent. The remainder of the response is probably dependent upon Ca^{2+} , specifically the Ca^{2+} influx as VIP gives a small increase in the intracellular $[Ca^{2+}]_i$, that is dependent upon extracellular Ca^{2+} .

Specificity of signaling or maintenance of microdomains arises from scaffolding proteins binding to specific signaling proteins forming multiprotein signaling complexes or signalosomes. As kinases such as PKA and PKC can phosphorylate a wide range of proteins and will phosphorylate the substrate in closest proximity, binding proteins keep the relevant kinase, substrate, and often phosphatase, as well as other signaling components in a preformed complex or a complex that forms upon agonist stimulation. As described by Beene and Scott (Beene and Scott 2007), "These signaling scaffolds serve as platforms for the integration and simultaneous dissemination of multiple signals. By sequestering a signaling enzyme to a specific subcellular environment, these proteins ensure that upon activation the enzyme is near its relevant targets. Thus scaffolds contribute to the spatiotemporal resolution of cellular signaling and are a key means by which a common signaling pathway can serve many different functions." For cAMP, the scaffolding proteins are known as A-kinase anchoring proteins (AKAP). These scaffolding proteins play a critical role in targeting and regulation of PKA-mediated phosphorylation, and, in addition, form multiprotein signalosomes that integrate cAMP-dependent pathways with other pathways.

There are over 50 members of the AKAP family (Beene and Scott 2007). Although structurally diverse, AKAPs are functionally similar and share three common features: 1) a PKA-anchoring domain, 2) sites to bind other signaling enzymes to form signalosomes, and 3) various targeting motifs (lipid modifications or protein-protein interactions) to direct the signalosomes to specific subcellular sites. cAMP activates PKA by binding to the RII subtype of the PKA regulatory (R) domain thus releasing the catalytic dimer from the regulatory dimer. The catalytic domain is then able to phosphorylate a substrate protein.

AKAPs also bind other signaling components (Figure 10). One signaling protein that can be bound by AKAPs is cAMP phosphodiesterase, the enzyme that terminates the action of cAMP by breaking it down to 5'AMP. This localized degradation of cAMP can contribute to localized cAMP gradients that further adds to specificity of signaling pathways. Another signaling protein often found in AKAP complexes is phosphatases that terminate the function of PKA by dephosphorylating the activated substrate.

In preliminary, unpublished data, three different types of AKAPs were detected in the lacrimal gland, AKAP150, WAVE1, and WAVE2 using western blotting analysis and immunofluorescence microscopy (Dartt DA Jonsson Invest Ophthalmol Vis Sci 2007 48:E-Abstract 1910). WAVE 1 and -2 were localized in myoepithelial cells in actin stress fibers and mitochondria, respectively. Additional AKAPs are undoubtedly present in the lacrimal gland contributing to the specificity of cAMP-dependent signaling.

Similarly to cholinergic agonists, in multiple tissues VIP is known to alter MAPK activity either by stimulating or inhibiting this enzyme (Burgering, Pronk et al. 1993; Cook and McCormick 1993; Wu, Dent et al. 1993; Chen and Iyengar 1994; Arslan and Fredholm 2000; Yamaguchi, Pelling et al. 2000; Stork and Schmitt 2002). In the lacrimal gland VIP inhibits MAPK activity as do compounds that increase cellular cAMP levels such as membrane permeable cAMP analogs and activators of adenylyl cyclase (Funaki, Hodges et al. 2007). Thus MAPK does not play an inhibitory role in VIP stimulated secretion, as it does for cholinergic agonists. As will be discussed in Section V, MAPK does play a role in the interaction of cAMP-dependent pathways with Ca^{2+} -PKC-dependent pathways that regulate protein secretion in vitro.

7. Neural Stimulation of Lacrimal Gland Secretion- Sympathetic Pathway

7.1. Stimulatory Nitric Oxide Signaling Pathway

Sympathetic nerves release the neurotransmitter norepinephrine that can activate α - and β -adrenergic signaling pathways. In the lacrimal gland, the predominant pathway activated by norepinephrine is the α_1 -adrenergic pathway (Thorig, Van Haeringen et al. 1983; Dartt, Rose et al. 1994). Cloned α_1 -adrenergic receptors activate PLC to produce InsP_3 and stimulate PLD activity. In contrast, in the lacrimal gland, α_1 -adrenergic receptors do not activate these pathways (Hodges, Dicker et al. 1992; Gromada, Jorgensen et al. 1995; Zoukhri and Dartt 1995). A clue to this unexpected response is that the subtype of functional α_1 -adrenergic receptors present in the lacrimal gland is the α_{1D} -adrenergic receptor, but not the more common α_{1A} - or α_{1B} -adrenergic receptor subtype. α_{1D} -Adrenergic receptors are normally found on the aorta or other large blood vessels and their cellular function is not well studied. In the lacrimal gland α_{1D} -adrenergic receptors activate three signaling pathways (Figure 11). First, stimulation of α_{1D} -adrenergic receptors induces endothelial nitric oxide synthase activity (eNOS) that was co-localized with caveolin in the basolateral membranes of lacrimal gland acinar cells (Hodges, Shatos et al. 2005). Using a variety of inhibitors Hodges et al found that activation of α_{1D} -adrenergic receptors stimulates eNOS to produce NO (Hodges, Shatos et al. 2005). In turn NO activates guanylate cyclase to produce cGMP and stimulate protein secretion. Surprisingly cGMP also activates the inhibitory MAPK pathway. In agreement with NO and cGMP mediating α_{1D} -adrenergic agonist stimulated secretion Gromada et al found that producing NO or activating cGMP stimulated protein secretion, but were not able to show that α_{1D} -adrenergic agonists used this pathway (Gromada, Jorgensen et al. 1995). Rather they suggested that α_1 -adrenergic agonists increased cyclicADP-ribose levels to increase $[\text{Ca}^{2+}]_i$ and activated guanylyl cyclase to produce cGMP to increase $[\text{Ca}^{2+}]_i$ (Gromada, Jorgensen et al. 1995; Jorgensen, Dissing et al. 1996). These studies, however, were not able to link stimulation of α_{1D} -adrenergic

receptors, production of cyclicADP-ribose, increase in the levels of cGMP, and an increase $[Ca^{2+}]_i$ and secretion together.

The molecular mechanisms that α_{1D} -adrenergic agonists use to activate eNOS have yet to be studied. In most tissues it is cholinergic agonists that activate NOS and they do so by increasing the $[Ca^{2+}]_i$ that activates Ca^{2+} /calmodulin-dependent protein kinases (Butt, Bernhardt et al. 2000). As α_{1D} -adrenergic agonists produce only a small increase in $[Ca^{2+}]_i$ this mechanism may not be used by α_{1D} -adrenergic agonists in the lacrimal gland.

7.2. Stimulatory and Inhibitory Protein Kinase C Signaling Pathways

A second pathway activated by α_{1D} -adrenergic agonists in the lacrimal gland involves PKC isoforms. Use of myristoylated pseudosubstrate inhibitors of individual PKC isozymes demonstrated that α_{1D} -adrenergic agonists activate PKC ϵ to stimulate protein secretion and PKC α and δ to inhibit secretion. The effector enzyme stimulated by α_{1D} -adrenergic agonists to activate PKC isoforms has not yet been identified, although it is known that α_{1D} -adrenergic agonists do not activate PLC or PLD (Hodges, Dicker et al. 1992; Gromada, Jorgensen et al. 1995; Zoukhri and Dartt 1995). These agonists could activate PLA $_2$ that would produce arachidonic acid and lysophospholipid to produce DAG that activate c- and n-PKC isoforms. Further investigation is warranted to determine if PKC ϵ functions in the stimulatory NO pathway and if PKC α and δ function in the inhibitory MAPK pathway or if these isoforms constitute their own separate pathways.

7.3. Inhibitory EGF Signaling Pathway

A third pathway activated by α_{1D} -adrenergic agonists is the EGF-dependent one leading to activation of MAPK and inhibition of secretion. α_{1D} -Adrenergic agonists, similarly to cholinergic agonists, activate MAPK that attenuates secretion. Unlike cholinergic agonists, α_{1D} -adrenergic agonists activate MAPK by transactivating the EGF receptor, a process known as receptor transactivation cascade (Chen, Hodges et al. 2006; Snider and Meier 2007). Receptor transactivation is the ability of one receptor to activate another through signaling events or cascades. In multiple tissues G protein-coupled receptors transactivate growth factor receptors such as the EGF receptor, platelet-derived growth factor (PDGF) receptor, or the nerve growth factor (NGF) receptor Trk (Snider and Meier 2007). Two mechanisms are used for receptor transactivation. One by activation of intracellular protein kinases such as Pyk2 and Src (Hodges, Rios et al. 2006) and the other by release of membrane-bound growth factors using membrane-bound matrix metalloproteinases (MMPs), a process known as the triple membrane passing signal (Prenzel, Zwick et al. 1999). Activation of growth factor receptors usually mediates mitogenesis extending the usually short-term effects of G protein-coupled receptors to longer-term effects on cell growth.

G protein-coupled receptor transactivation of EGF receptors is often mediated by cleavage of the membrane-bound growth factor, with heparin bound- (HB)-EGF and transforming growth factor α (TGF α) being the most common growth factors released. The EGF family of growth factors is a family of membrane-bound proteins with an extracellular domain of varying length that contains the active 6 kDa GF binding domain. Extracellular cleavage of these growth factors releases the extracellular domain with its active motif that can then bind to and activate the EGF receptor. This process is known as ectodomain shedding. The release of the extracellular domains into the extracellular space occurs by the stimulation of plasma membrane located MMPs, including MMP-2, -3, and -9 and ADAM10, 15, and 17 (Snider and Meier 2007). The molecular mechanism by which the G protein-coupled receptor activates the MMP is unknown. The resultant effect, however, is the release of the

active domain of the growth factor that binds to the EGF receptor activating it resulting in a phosphorylation cascade leading to stimulation of MAPK activity.

EGF receptor transactivation has been especially well studied in carcinoma cells in which the EGR receptor is over expressed (Snider and Meier 2007). In primary untransformed cells, the studies are more limited. In these latter cells, the result of transactivation can be cell growth and differentiation, as well as smooth muscle contraction and protein secretion.

In the lacrimal gland, α_{1D} -adrenergic agonists induce a receptor transactivation cascade (Chen, Hodges et al. 2006). These agonists activate the disintegrin and matrix metalloproteinase (MMP) ADAM17, also known as tumor necrosis factor- α converting enzyme (TACE) to release EGF by ectodomain shedding. The released EGF activates the EGF receptor dimer to attract and phosphorylate the adapter proteins Grb2 and Shc. Activation of these proteins stimulates SOS to induce Ras that in turn activates Raf (MAPKKK), MEK (MAPKK), and MAPK. Activated MAPK then phosphorylates unknown substrates in the cytoplasm that inhibit secretion.

Transactivation of the EGF receptor by α_{1D} -adrenergic agonists in the lacrimal gland is unique on several different levels. First, α_{1D} -adrenergic agonists activate the MMP, ADAM17 rather than ADAM 10 an MMP known to cause shedding of EGF and betacellulin (Sahin, Weskamp et al. 2004). Second, α_{1D} -adrenergic agonists release EGF, rather than the usual growth factors HB-EGF and TGF α . Third, activation of the EGF-dependent MAPK signaling pathway leads to a short-term process, protein secretion. Finally, α_{1D} -adrenergic agonist activation of MAPK induces an inhibitory process, attenuation of protein secretion.

Similar to cholinergic agonists, α_{1D} -adrenergic agonists in the lacrimal gland activate both stimulatory and inhibitory pathways. However, the pathways activated differ between these two agonists. Cholinergic agonists activate PLC via Ca^{2+} and PKC α , δ , and ϵ to stimulate secretion and activate MAPK via two pathways, PLD and Pyk2/Src to attenuate secretion. α_{1D} -Adrenergic agonists do not use the PLC and PLD pathways, instead they activate two pathways NO/cGMP and PKC ϵ to stimulate secretion and one pathway EGF receptor transactivation/MAPK to inhibit secretion. Based on the activation of separate, different signaling pathways by cholinergic and α_{1D} -adrenergic agonists, the resultant lacrimal gland protein secretion is additive that is greater than either response by itself.

7.4. Stimulatory EGF Signaling Pathway

Exogenously added EGF itself, surprisingly, stimulates lacrimal gland protein secretion (Tepavcevic, Hodges et al. 2003). The EGF commercially available and used in these studies is the 6kDa active site, found only in the submaxillary salivary gland where it is stored in secretory vesicles and released by exocytosis (Rougeot, Rosinski-Chupin et al. 2000). EGF presumably activates PLC γ , which then increases the intracellular $[Ca^{2+}]$ and activates the PKC isoforms PKC α and δ to stimulate secretion.

EGF activation of PI3K and MAPK, measured by using inhibitors of these pathways, does not alter EGF-stimulated secretion. Importantly, EGF does not use MAPK to stimulate secretion, as activation of this enzyme has been shown to attenuate cholinergic and α_1 -adrenergic agonist stimulation of secretion.

When EGF was added with the cholinergic agonist carbachol or the α_1 -adrenergic agonist phenylephrine, the resultant secretion was less than additive suggesting that the EGF/cholinergic and EGF/ α_1 -adrenergic pathways overlapped. For the cholinergic pathway, EGF and cholinergic agonists both increase the intracellular $[Ca^{2+}]$ and activate PKC α and δ to stimulate secretion, although there are several differences in the details of the pathways

activated by the two agonists including: 1) cholinergic agonists increase Ca^{2+} about 3-fold greater than EGF and 2) cholinergic agonists, but not EGF, use PKC ϵ to stimulate secretion. Thus evidence suggests that cholinergic agonists use similar, but not identical pathways for secretion. For the α_1 -adrenergic pathway and EGF, the experimental evidence for the use of similar pathways is less convincing. Although EGF and α_1 -adrenergic agonists both cause a small increase in the intracellular $[\text{Ca}^{2+}]$, the activation of PKC isoforms is different. α_1 -Adrenergic agonists activate PKC ϵ to stimulate secretion and PKC α and $-\delta$ to inhibit secretion, whereas EGF uses PKC α and $-\delta$ to stimulate secretion. It is possible, but not yet investigated, that EGF activates NO/cGMP as do α_1 -adrenergic agonists and these actions could contribute to the overlap of the signaling pathways. A final inconsistency to be addressed is that α_1 -adrenergic agonist release of the ectodomain of EGF and its activation of the EGF receptor attenuates secretion, whereas exogenous addition of EGF stimulates secretion. One possible explanation for this is the existence of signalosomes. These are multiple signaling proteins (enzymes as well as their substrates) held in proximity to each other by binding to a scaffold protein. Thus there could exist a signalosome with the α_1 -adrenergic receptor, matrix metalloproteinase, EGF receptor, Shc, Grb2, SOS, Ras, Raf, Mek, MAPK, and a MAPK substrate that inhibits secretion. A second signalosome could contain the EGF receptor, PLC γ , PKC α and $-\delta$, and a PKC substrate(s) that stimulate secretion. α_1 -Adrenergic agonists would activate the first type of signalosome thereby attenuating secretion, whereas exogenously added EGF would stimulate the second variety of signalosome and induce protein secretion. An alternative possibility is that the EGF ectodomain released by α_1 -adrenergic agonist stimulation activates dissimilar residues on the EGF receptor than exogenously added 6 kDa-EGF and this differential activation would attract disparate adapter proteins that would inhibit or stimulate secretion, respectively.

8. Neural Stimulation of Secretion-Interaction of Cholinergic and α_1 -Adrenergic Pathways with the VIP Pathway

Several laboratories demonstrated that the simultaneous addition of a Ca^{2+} /PKC dependent agonist such as cholinergic agonists, α_1 -adrenergic agonists or EGF with a cAMP-dependent agonist such as VIP caused synergism or potentiation of protein secretion (Dartt, Baker et al. 1984; Mauduit, Herman et al. 1987; Dartt, Ronco et al. 1988). Potentiation also occurred when the second messengers such as the intracellular $[\text{Ca}^{2+}]$ or PKC activity were increased at the same time as cellular levels of cAMP were increased (Mauduit, Herman et al. 1987; Funaki, Hodges et al. 2007). Potentiation of secretion could occur by potentiation of the: 1) increase in the intracellular $[\text{Ca}^{2+}]$, 2) activation of PKC isoforms, or 3) increase in cellular cAMP levels. In 1988, my laboratory demonstrated that potentiation of the intracellular $[\text{Ca}^{2+}]$, PKC activity, or cAMP levels did not occur (Dartt, Ronco et al. 1988). Recently we discovered that an inhibition of MAPK activity causes potentiation of protein secretion by relieving its attenuating effects on agonist-stimulated secretion (Funaki, Hodges et al. 2007). MAPK activity was inhibited by increasing cellular cAMP levels with VIP, adding forskolin that activates adenylyl cyclase, or adding membrane-permeant cAMP analogs (Figure 12). When cellular cAMP levels are increased simultaneously with addition of Ca^{2+} /PKC-dependent agonists, the Ca^{2+} /PKC-dependent agonist induced activation of MAPK is prevented and can no longer attenuate secretion. The effect was robust as it occurred when cellular cAMP levels were increased by a receptor-mediated stimulus, activation of adenylyl cyclase, or by increasing cellular cAMP levels by membrane permeant cAMP analogs. Potentiation additionally occurred with three Ca^{2+} /PKC-dependent agonists, cholinergic agonists, α_1 -adrenergic agonists or EGF.

9. Mechanism of Lacrimal Gland Protein Secretion

9.1. Newly Identified Lacrimal Gland Secretory Proteins

One of the main functions of the lacrimal gland is to synthesize and secrete proteins into the tear fluid. These proteins play critical roles in protecting the cornea and conjunctiva from challenges in the external environment, as well as in regulating the function of these epithelia. Not surprisingly, the lacrimal gland secretes a myriad of proteins many of which are listed in Dartt and Sullivan (Dartt and Sullivan 2000). Several secretory proteins have been newly identified in the lacrimal gland.

Sanghi et al have identified and characterized lacritin, a 12.3 kDa glycoprotein secreted predominantly by the lacrimal gland and hence found in tears (Sanghi, Kumar et al. 2001). Even though lacritin is secreted by the lacrimal gland, it also alters lacrimal gland function by stimulating secretion (Sanghi, Kumar et al. 2001). Lacritin also stimulate corneal epithelial cell proliferation and is protective against pro-inflammatory cytokines (Wang, Wang et al. 2006; McKown, Wang et al. 2008).

Although the lacrimal gland is characterized as serous and not mucous, this gland does synthesize and secrete mucins. The large gel forming secretory mucin MUC5B has been consistently identified in the cytoplasm of lacrimal gland acinar cells as has the smaller secretory mucin MUC7 (Jumblatt, McKenzie et al. 2003; Paulsen 2006). It is likely that these mucins are actually secreted by the lacrimal gland into the tear film. If so it will be interesting to determine their role in the tear film and their effect on the ocular surface.

The lacrimal gland synthesizes a plethora of proteins that are secreted onto the ocular surface and function to regulate and maintain the corneal and conjunctival epithelia. One function of lacrimal gland proteins is to protect the ocular surface from bacterial invasion. Two proteins newly identified in the human lacrimal gland are lipopolysaccharide (LPS)-binding protein, LBP, and CD14 (Blais, Vascotto et al. 2005). These proteins complement the LPS receptor complex expressed by the corneal epithelium to trigger an immune response in the presence of LPS.

Two other proteins related to protection of the ocular surface by its innate immune defense system are surfactant proteins A and D (SP-A and SP-D). These proteins are members of the collectin family. Using RT-PCR, western blotting analysis, and immunofluorescence microscopy SP-A and SP-D were detected in both the lacrimal gland and in tears (Brauer, Kindler et al. 2007; Herias, Hogenkamp et al. 2007). SP-B and SP-C were also detected (Brauer, Johl et al. 2007). These surfactant proteins could function in lowering the surface tension of the tears as well as in innate immune defense.

Chitotriosidase is a chitinolytic enzyme expressed in macrophages and neutrophils (Hall, Morroll et al. 2008). This enzyme is expressed at high levels in the mouse and human lacrimal gland. Chitotriosidase has anti-fungal and anti-bacterial activity. It functions complementarily with lysozyme as Chitotriosidase has a different antimicrobial spectrum than lysozyme.

Based on the transcriptome described by Ozyildirim that indicated a plethora of lacrimal gland proteins yet to be identified, it is not surprising that new antibacterial proteins are being continuously found in the lacrimal gland and tears (Ozyildirim, Wistow et al. 2005). New secretory protein present in the lacrimal gland and in tears will continue to appear.

9.2. Overview of Protein Secretory Mechanisms

Lacrimal gland proteins can be secreted by at least three different mechanisms, exocytosis (meaning the regulated fusion of stored secretory granules with the apical membrane), transcytosis, and ectodomain shedding. Although specific proteins are secreted by each of these pathways, a single protein can use multiple pathways under different conditions. A complex series of intracellular trafficking pathways elucidated by Mircheff and his collaborators over the years have provided the framework for understanding the complex organization of vesicles in the trafficking pathways of lacrimal gland acinar cells and how these pathways are altered with stimulation for different proteins (Wang, Chiu et al. 2007; Wang, Chiu et al. 2007).

9.3. Exocytosis

The major pathway for secretion is by exocytosis across the apical membrane of proteins produced, packaged in secretory granules, and stored in the acinar cells (Figure 13). Lacrimal gland secretory proteins such as lactoferrin, lysozyme, β -hexosaminidase, secretory mucins, and peroxidase are secreted by stimulus induced exocytosis. As reviewed by Wu et al, there are different types of trafficking effectors that control the exocytotic process (Wu, Jerdeva et al. 2006). These effectors include targeting and specificity factors such as rabs and SNAREs as well as transport factors including microtubules, actin filaments, and motor proteins. Exocytosis occurs with stimulation of cellular signaling pathways by the agonists described in sections V, VI, and VII. All agonists that stimulate lacrimal gland increase in the intracellular $[Ca^{2+}]$ by at least a minimal amount (Sundermeier, Matthews et al. 2002). The increase in intracellular $[Ca^{2+}]$ causes the fusion of mature secretory vesicles with the apical membrane. Wu et al have formulated the following hypothesis for the lacrimal acinar exocytotic secretory pathway (Wu, Jerdeva et al. 2006) (Figure 13). Under unstimulated conditions, mature secretory vesicles enriched in Rab3D, a member of the small Ras-like GTPases, are maintained beneath a dense apical actin filament network adjacent to the apical membrane. These mature secretory vesicles are maintained in this localization by cytoplasmic dynein, a motor protein that regulates the movement of vesicles along microtubules. Another population of secretory vesicles that are recruitable are found in the cytoplasm. These recruitable secretory vesicles are enriched in VAMP2, which is a member of the SNARE family of proteins that regulate the fusion of the secretory vesicle membrane with the plasma membrane. Upon stimulation the apical actin filaments thin and Rab3D is lost from primed individual secretory vesicles that have been localized in clusters and surrounded by a basket-like network of actin and non-muscle myosin II filaments. Cholinergic and α_1 -adrenergic agonists activate PKC ϵ , which binds to actin and phosphorylates proteins involved in actin filament thickening. The class V myosin motor, myosin 5c, colocalizes with Rab3D and plays a role in the association of actin coats around the fusing mature secretory vesicles during exocytosis (Marchelletta, Jacobs et al. 2008). The interaction of actin filaments and non-muscle myosin II helps the aggregated secretory vesicles to fuse in the cytoplasm and promotes the migration of the cluster toward the apical membrane. An increased turnover of the apical actin network increases the availability of sites on the apical membrane for the cluster of fused secretory vesicles to interact, fuse, and release their contents. SNAREs interact with the mature secretory vesicles, but they specific ones have yet to be identified. In addition the recruitable secretory vesicles are recruited to the apical membrane by moving along the microtubules tracks powered by dynein. For these vesicles, the v-SNARE VAMP2 interacts with the t-SNARE syntaxin-3 to facilitate the fusion of these smaller recruitable vesicles with the apical membrane. Another laboratory demonstrated that VAMP8 is a v- SNARE in the lacrimal gland that may interact with syntaxin4 and SNAP-23 to regulate exocytosis (Wang, Shi et al. 2007).

9.4. Transcytosis

One important protein secreted by the lacrimal gland, secretory IgA, uses the transcytotic, ectodomain shedding, and exocytosis pathways. Secretory IgA is produced by the wet-surfaced epithelia of the body as an immunospecific barrier to prevent microbial infection (Kaetzel, Robinson et al. 1991). In the lacrimal gland IgA⁺ plasmacytes produce the majority of dimeric IgA that appears in the tears (Franklin, Kenyon et al. 1973; Allansmith, Kajiyama et al. 1976). Secretory IgA is synthesized and secreted by a complex cell trafficking process. As summarized by Evans the receptor of dimeric IgA is the polymeric immunoglobulin receptor (pIgR), which is a single transmembrane receptor (Figure 14) (Evans, Zhang et al. 2008). This receptor is synthesized in the endoplasmic reticulum and modified in the Golgi apparatus of lacrimal gland acinar cells. It is then delivered by the trans-Golgi network to the basolateral membrane where it is inserted into the membrane with its large ligand binding domain extending into the extracellular space. The pIgR binds to dimeric IgA or IgM and is endocytosed (with or without ligand) and transported through a series of endomembrane compartments across the cell to the apical membrane. This process is known as transcytosis. At apical surface the extracellular domain of pIgR that is bound to the dimeric IgA is proteolytically cleaved and secretory IgA is released into the lumen and the lacrimal gland fluid. If the pIgR does not bind dimeric IgA at the basolateral membrane it is still transcytosed and inserted into the apical membrane. The extracellular domain that is then cleaved and released by ectodomain shedding into the lumen is known as secretory component. The secretagogue regulation of sIgA and secretory component production has been studied by Sullivan and colleagues (Sullivan, Kelleher et al. 1990) (Gao, Lambert et al. 1995; Sullivan, Wickham et al. 1998); and is complex. Compared to secretion of proteins by exocytosis, which is regulated on the time scale of seconds to minutes by neurotransmitters, secretion of sIgA is regulated by the rate of synthesis. Secretory component synthesis is stimulated on the time scale of hours by androgens and other compounds (Sullivan, Kelleher et al. 1990). In contrast, the regulation of ectodomain shedding of secretory component has yet to be studied.

Recently Evans et al found that the pIgR can be localized to the exocytotic pathway through its interaction with Rab3D (Evans, Zhang et al. 2008). Rab3D appears to traffick pIgR into the regulated exocytotic pathway either directly from the trans-Golgi network or indirectly from the transcytotic pathway. As with the regulated exocytotic pathways described previously, cholinergic agonists decrease the association between Rab3D and pIgR consistent with the stimulation of secretory component release into the lumen. Either Rab3D could function to retain pIgR in the mature secretory vesicles or pIgR could be involved in the targeting of Rab3D to the mature secretory vesicles. Interestingly exogenously added dIgA increases the intracellular [Ca²⁺] similarly to the cholinergic agonists underlying the important role of Ca²⁺ in exocytosis (Sundermeier, Matthews et al. 2002). (Figure 14)

Another protein secreted by lacrimal gland acini, prolactin uses both the transcytotic and the exocytotic pathways. As summarized by Wang et al lacrimal gland acinar and duct cells can regulate their local extracellular milieu by secretion of cytokines and growth factors by a paracrine secretory process that has much in common with the transcytosis pathway (Wang, Chiu et al. 2007). Newly synthesized cytokines and growth factors emerge from the endoplasmic reticulum, Golgi complex, and trans-Golgi network as do other synthetic proteins. They are directed into a network of endomembrane compartments that are linked to each other and the plasma membrane by a population of transport vesicles that comprise a constitutive, transcytotic paracrine apparatus. The transport vesicles arising from the endosomes fuse with the basolateral membrane to insert pIgR to be available to bind dimeric IgA and release their cargo that can include cytokines and growth factors.

One of these growth factors that could be in the cargo is prolactin that is produced by the pituitary gland. Prolactin can be taken up by endocytosis by the lacrimal gland, but also can be synthesized by the lacrimal gland acinar and duct cells. In non-pregnant females and in males (low prolactin levels) both synthesized and endocytosed prolactin is localized in the apical cytoplasm consistent with secretion by the regulated exocytotic pathway. Cholinergic agonists can then stimulate prolactin secretion via the exocytotic pathway, consistent with cholinergic regulation of exocytosis (Wang, Chiu et al. 2007). During pregnancy (high prolactin levels) the localization of prolactin changes to the basal cytoplasm consistent with paracrine secretion using the transcytotic pathway. When prolactin is secreted from the basal side it appears to enhance the transcytotic paracrine apparatus that trafficks sIgA into the apical secretion and hence the tears. The elevated levels of prolactin suppress traffic of secretory proteins into the regulated exocytotic apparatus into the endosomes of a paracrine pathway that intersects the constitutive transcytotic pathway (Wang, Chiu et al. 2007; Wang, Chiu et al. 2007).

9.5. Ectodomain Shedding

In mast cells about 2% of proteins can be secreted by ectodomain shedding. A common stimulus of ectodomain shedding is phorbol 12-myristate-13-acetate (PMA), a activator of c- and nPKC isoforms. As previously mentioned secretory IgA and the EGF family of growth factors are secreted by ectodomain shedding. EGF and its family members including transforming growth factor (TGF) α , heparin-binding EGF (HB-EGF), and heregulin are single transmembrane proteins that are inserted into plasma membrane with an extracellular domain that includes the 6 kDa active fragment (6kDa EGF). In all cells except those of the male submaxillary gland EGF is a transmembrane protein. In the former the 6kDa EGF is stored in secretory granules and released by exocytosis. Although all of the members of the EGF family named above are present in the lacrimal gland, only EGF has been localized and its secretion studied. EGF is present in the basolateral and apical membranes (Chen, Hodges et al. 2006). As described in section VII, α_{1D} -adrenergic agonists activate the matrix metalloproteinase ADAM17 to cleave the extracellular domain of the EGF releasing the active fragment so that it can bind to the EGF receptor to activate it and attenuate regulated exocytotic secretion.

10. Neural Regulation of Lacrimal Gland Electrolyte and Water Secretion

Although protein secretion is easily studied in a variety of in vitro preparation of lacrimal gland tissue, in vitro study of electrolyte and water secretion has been difficult until recent developments. Selvam et al developed a method to culture lacrimal gland acinar cell monolayers on polyester membrane scaffolds (Selvam, Thomas et al. 2007). Using rabbit lacrimal gland acinar cells Selvam et al (2007) confirmed the previous models of electrolyte and water secretion hypothesized from studies using the in vivo gland or in vitro study of transport protein activity (Selvam, Thomas et al. 2007). The currently proposed mechanism for lacrimal gland acinar cell fluid secretion is that the basolateral Na^+, K^+ -ATPase produces the energy to drive the efflux of Na^+ and influx of K^+ across the basolateral membrane against their electrochemical gradients (Figure 15). The Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers and the $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ symporter use the energy of the favorable inward Na^+ gradient to drive Cl^- influx. This Cl^- influx establishes a favorable gradient for Cl^- efflux across the apical membrane through Cl^- selective channels into the lumen generating a lumen negative transepithelial potential difference. This potential difference drives the flux of Na^+ from the basal side into the apical secretion through the paracellular pathway. K^+ selective channels allow K^+ to recycle across the basolateral membranes so that acini can produce a Na^+-Cl^- rich fluid. The cholinergic agonist carbachol stimulates fluid secretion by activating apical Cl^- channels (Findlay and Petersen 1985; Saito, Ozawa et al. 1985; Evans, Marty et al. 1986), apical K^+ channels (Lechleiter, Dartt et al. 1988) and basolateral Na^+-H^+ exchangers

(Saito, Ozawa et al. 1987; Lambert, Bradley et al. 1991) as well as by increasing the translocation of the Na^+, K^+ -ATPase from vesicles in the cytoplasm to the basolateral membrane (Yiu, Lambert et al. 1991). Stimulated fluid secretion could occur by active electrogenic secretion of an anion, probably Cl^- or active electrogenic absorption of a cation, probably Na^+ . Neither of these processes could be excluded by the study of Selvam et al. who measured short circuit current as an index of fluid secretion. Use of the acinar cell monolayer will be useful to study lacrimal gland fluid secretion especially to investigate the signaling pathways activated by the neurotransmitters acetylcholine, VIP, and norepinephrine to determine if similar pathways activate both regulated exocytotic secretion of proteins and electrolytes/water. Since cholinergic and α_{1D} -adrenergic agonists use different signaling pathways to stimulate protein secretion, but appear to use similar pathways to activate basal ion channels (Hodges, Dicker et al. 1992; Satoh, Sano et al. 1997), it will be important to determine which signaling pathways are used for ion and water secretion.

The molecular mechanisms responsible for protein and fluid secretion by lacrimal gland duct cells are woefully understudied. Two new methods have been employed to study duct cell function in vitro. First Ubels et al (Ubels, Hoffman et al. 2006) used laser capture microdissection to isolate duct cells from the rat lacrimal gland. RNA was isolated from the ducts and analyzed by microarray. Mircheff (Mircheff 1989) hypothesized that transepithelial electrolyte transport in duct cells differs from that in acinar cells by the K^+ channels being placed in the apical membrane along with the Cl^- channels causing the formation of a $\text{K}^+ \text{Cl}^-$ rich fluid. Ubels et al examined the K^+ and Cl^- transport channels differentially expressed in duct compared to acinar cells (Ubels, Hoffman et al. 2006). In duct cells the $\text{Na}^+ \text{K}^+ 2\text{Cl}^-$ symporter NKCC1, $\text{Na}^+ \text{K}^+$ -ATPase, and the M_3 muscarinic receptor were located on the basolateral membrane of duct cells, whereas the $\text{K}^+ \text{Cl}^-$ symporter KCC1, the intermediate conductance calcium-activated K^+ channel $\text{IK}_{\text{Ca}1}$, the cystic fibrosis transmembrane regulator (CFTR Cl^- channel, and $\text{CIC}3$ Cl^- channel were apically localized. The intermediate conductance calcium-activated K^+ channel $\text{IK}_{\text{Ca}1}$ was also identified in the lacrimal gland by Thompson-Vest et al (Thompson-Vest, Shimizu et al. 2006). The large-conductance calcium activated channel (BK_{Ca}), shown by Lechleiter to function in lacrimal gland acinar cells, was not on the array used (Lechleiter, Dartt et al. 1988). (Figure 16). Thus confirming and extending the hypothesis of Mircheff, the $\text{Na}^+ \text{K}^+ 2\text{Cl}^-$ symporter NKCC1 and $\text{Na}^+ \text{K}^+$ -ATPase located on the basal side of the duct cells provide the energy to load the cells with K^+ and Cl^- , which are then secreted into the duct lumen by the $\text{K}^+ \text{Cl}^-$ symporter KCC1, the intermediate conductance calcium-activated K^+ channel $\text{IK}_{\text{Ca}1}$, the CFTR Cl^- channel, and $\text{CIC}3$ Cl^- channel (Mircheff 1989). Duct cell electrolyte and water secretion is stimulated by cholinergic agonists that bind to the M_3 muscarinic receptors on the basal membranes increasing the intracellular $[\text{Ca}^{2+}]$ that activates the $\text{IK}_{\text{Ca}1}$ K^+ channel in the apical membrane to initiate the secretory process. The BK_{Ca} K^+ if present channels would also be activated. Cl^- follows the K^+ using the apical CFTR and $\text{CIC}3$ Cl^- channels.

Functional confirmation of the mechanism for K^+ and Cl^- transport by lacrimal gland duct cells came from the work of Toth-Molnar et al who developed a rapid method to isolate large quantities of intact ducts from the rabbit lacrimal gland (Toth-Molnar, Venglovecz et al. 2007). Use of fluorescent dyes to measure the intracellular pH and $[\text{Ca}^{2+}]$ of the ducts showed that cholinergic agonists increased the intracellular $[\text{Ca}^{2+}]$ of duct cells that in turn stimulated the $\text{Na}^+ \text{H}^+$ exchanger followed by the $\text{Cl}^-/\text{HCO}_3^-$ exchanger both on the basal membrane as occurs in acinar cells. This would increase the Cl^- level in the cells to stimulate apical Cl^- efflux. Not shown by this method, but in agreement with Ubels the increased intracellular $[\text{Ca}^{2+}]$ would activate the apical $\text{IK}_{\text{Ca}1}$ K^+ channel to cause apical K^+ efflux resulting in the $\text{K}^+ \text{Cl}^-$ rich duct cell secretion that would modify the $\text{Na}^+ \text{Cl}^-$ acinar cell secretion resulting in a Na^+ , K^+ , and Cl^- containing lacrimal gland fluid that exits onto

the ocular surface. Use of the isolated ducts holds great promise for further study of the ionic mechanism of duct cell secretion, identification of the stimuli of duct secretion of electrolyte and water as well as proteins, and characterization of the signaling pathways used.

11. Neural Regulation of Lacrimal Gland Function and Its Alteration in Disease

11.1. Overview

Neural regulation of lacrimal gland function is complex with the first step in regulation being activation of sensory nerves in the cornea and conjunctiva, the second being stimulation of the efferent parasympathetic and sympathetic nerves, the third being activation of the cellular signaling pathways in acinar and duct cells, and the final step being the secretion of proteins, electrolytes, and water. An impairment of lacrimal gland function and resultant ocular surface diseases could occur at any of these steps that in themselves comprise multiple steps or mechanisms. Select examples of dry eye disease resulting from alterations in these four steps will be presented. Additional examples can be found in Pflugfelder, Stern, and Beuerman and Dartt (Dartt 2004; Pflugfelder, Stern et al. 2004).

11.2. Impairment of Sensory Nerve Function

Although not specific to lacrimal gland function, an alteration in the sensitivity of corneal nerves can cause dry eye in part by blocking lacrimal gland protein, electrolyte, and water secretion. A decrease in the sensitivity of sensory nerves can occur in aging, diabetes, and LASIK surgery. There are a few studies on the alteration of corneal sensitivity with aging. In humans one study reported that corneal sensitivity decreased with increasing age, as measured with a non-contact corneal esthesiometer, which is a superior method than using the Cochet-Bonnet esthesiometer (Murphy, Patel et al. 2004) (reference from aging study). The change in sensitivity in humans did not appear to be due to a change in the density or orientation of subbasal nerves in the central cornea (Erie, McLaren et al. 2005). Unfortunately tear secretion was not measured in either of these studies. A definitive connection between a decrease in corneal sensitivity and tear secretion would be important to link these two processes.

An alteration in corneal sensitivity has been detected in patients with diabetes. Using a non-contact esthesiometer, Cousen et al (Cousen, Cackett et al. 2007) and Murphy et al (Murphy, Patel et al. 2004) each found a decrease in corneal sensitivity in patients with diabetes. A similar decrease in sensitivity was found by Nuho et al (Nuho, Subekti et al. 2004). Cousen et al correlated the decrease in corneal sensitivity with a decrease in tear production as measured by the Schirmer test with topical anesthesia (Cousen, Cackett et al. 2007). These alterations could account in part for the increased risk that diabetic patients have for developing corneal lesions such as superficial punctate keratitis, recurrent corneal erosions, persistent epithelial defects, and microbial keratitis.

LASIK can also cause a decrease in tear production associated with impaired sensory nerve activity. In LASIK the corneal sensory nerves are severed when the corneal flap is created leaving the central cornea devoid of nerves except those that course through the intact hinge. Most individuals develop a temporary dry eye after LASIK resulting from the trauma of the procedure and the severing of the sensory nerves. This dry eye consists of a decrease in: 1. corneal sensitivity, 2. number of filled goblet cells, 3. Schirmer test with and without anesthesia, and 4. the tear break-up time (Konomi, Chen et al. 2008). Also occurring is an increase in rose Bengal staining and in conjunctival squamous metaplasia. The appearance of this temporary dry eye occurs because of a decrease in lacrimal gland and goblet cell secretion. In a small percentage of individuals this dry eye becomes chronic and is

associated with a persistent decrease in tear volume and break up time. Two factors could explain this chronic dry eye. First, the regrowth of the corneal nerves has been impaired leading to a decrease in lacrimal gland secretion as well as the painful sensations of aberrantly regenerating corneal nerves (Belmonte 2007). These sensations mimic the symptoms of dry eye. Second the ocular surface has not been able to recover from the temporary changes in lacrimal gland, goblet cell, hence tear film secretion after surgery and remains damaged.

11.3. Impairment of Efferent Nerve Function

Peripheral neuropathy is an important component of the pathology of Sjogren's syndrome (Sorajja, Poirier et al. 1999; Hocevar, Tomsic et al. 2003). The trigeminal nerve is one of the most commonly affected cranial nerves in primary Sjogren's syndrome (Gemignani, Marbini et al. 1994; Barendregt, van den Bent et al. 2001). Using a modified Belmonte non-contact esthesiometer, Tuisku et al found that a mechanical hypersensitivity in corneal nerves of individuals with Sjogren's syndrome compared to normal age sex matched controls (Tuisku, Kontinen et al. 2008). The hypersensitivity correlated with an increase in the subjective symptoms of the patients compared to the controls. When the corneal nerves were examined by scanning slit confocal microscopy, there was no difference in the density of the nerves, but there was an increase in nerve sprouts and dendritic antigen presenting cells between patients and controls. These findings suggest that patients with Sjogren's syndrome have a neuropathic corneal mechanical hypersensitivity that could be induced by inflammation or by the aberrant firing of nerves attempting to regenerate as suggested by Belmonte (Belmonte 2007).

In spite of the increased corneal nerve sensitivity, patients with Sjogren's syndrome had a decreased Schirmer test compared to controls. This suggests that the impairment of tear production and lacrimal gland fluid secretion in Sjogren's syndrome occurs at a different step other than activation of corneal nerves in the neural regulation of lacrimal gland secretion (Figure 17). Possible mechanisms for inhibition of secretion in the setting of Sjogren's syndrome come from animal studies. In the MRL/MpJ-Fas^{lpr} mouse model of Sjogren's syndrome, Zoukhri et al demonstrated that there is not an impairment of efferent nerve stimulation of protein secretion, but an increase in the intracellular Ca²⁺ response to exogenously added neurotransmitters as would be expected from a denervation-like supersensitivity (Zoukhri, Hodges et al. 1998; Zoukhri and Kublin 2001; Zoukhri, Hodges et al. 2002). The impaired neural stimulation of secretion arose from the presence of proinflammatory cytokines such as IL-1 β preventing the release of neurotransmitters from the nerve terminals. As inflammation of the lacrimal gland is a hallmark of Sjogren's syndrome in patients, the blockage of neurotransmitter release could account for the decrease in lacrimal gland secretion indicated by a decrease in tear production in spite of the increased afferent neural stimulation. Sjogren's syndrome is a complex autoimmune disease, thus additional pathological changes could also contribute to the impairment of lacrimal gland secretion.

11.4. Impairment of Lacrimal Gland Signaling Pathways

In a mouse model of aging neural stimulation of protein secretion is disrupted early in the aging process, as in the MRL/MpJ-Fas^{lpr} mouse model of Sjogren's syndrome (Rios, Horikawa et al. 2005). Along with the loss of neural stimulation of protein secretion is a loss of exogenously added neurotransmitter stimulation of secretion that does not occur in the mouse model of Sjogren's syndrome. This finding suggests that not only is neurotransmitter release impaired with aging, but, in addition, the signaling pathways activated by cholinergic and α_1 -adrenergic agonists are deficient. The components of the signaling pathways that have been affected have not yet been identified. As about 5-10% of the population suffers

from dry eye, it is possible that genetic polymorphisms in genes encoding proteins that comprise the signaling pathways could predispose individuals to dry eye especially as they age.

Another cause of dry eye is the use of anti-cholinergic medications. A wide variety of medication including phenothiazines, anti-histamines, tricyclic antidepressants, and atropine are anti-cholinergic. The anti-cholinergic effect of these medications would prevent the binding of acetylcholine with its M_3 muscarinic receptor and the activation of its signaling pathway.

A cause of altered lacrimal gland secretion in Sjogren's syndrome, in addition to the blocked release of neurotransmitters, is the presence of function blocking M_3 muscarinic antibodies (Bacman, Berra et al. 2001). These antibodies bind to the muscarinic receptor and prevent its activation by acetylcholine.

11.5. Impairment in Secretion of Proteins, Electrolytes, and Water

Off target drug effects are a source of dry eye disease with diuretics being one class of these drugs. The diuretic furosemide, which inhibits the $Na^+-K^+-2Cl^-$ transporter, an enzyme responsible for Cl^- secretion, can cause the side effect of dry eye.

12. Potential Directions for Future Studies

Proper functioning of each component of the lacrimal gland functional unit including the afferent sensory nerves from the ocular surface, the efferent parasympathetic and sympathetic nerves that innervate the lacrimal gland, and the lacrimal gland acinar and ductal cells is essential for secretion of lacrimal gland fluid in appropriate amount and composition thus ensuring a healthy ocular surface. Over the past few years significant progress has been made describing the activation of sensory nerves of the ocular surface as well as in identifying the signaling pathways of lacrimal acinar cells, the cellular trafficking mechanisms, and the components involved in the secretory process itself. Several areas of lacrimal gland function are in need of future research. The first area is to determine the cellular pathways that regulate electrolyte and water secretion stimulated by neurotransmitters. The development of an artificial lacrimal gland by Selvam et al that contains a monolayer of acinar cells is a breakthrough in the ability to measure electrolyte and water secretion in vivo (Selvam, Thomas et al. 2007). This preparation could be used to determine if the agonists that stimulate protein secretion, use the same signaling pathways to induce fluid secretion. Both pharmacological and molecular inhibitors could be used.

A second area of future research is to characterize duct cell function. The use of the isolated duct cell preparation developed by Toth-Molnar can support investigation of the mechanisms of duct cell electrolyte, water and protein secretion, identification of the proteins secreted by duct cells, and determination of the agonists that stimulate secretion, as well as the signaling pathways used by these stimuli (Toth-Molnar, Venglovecz et al. 2007).

A third area is identification of novel proteins secreted by the lacrimal gland. Ozyildirim et al have characterized the transcriptome of the human and mouse lacrimal gland and found that the lacrimal gland has a large number of rare transcripts and hypothetical proteins predicted from genome sequence (Ozyildirim, Wistow et al. 2005). Some of these could be secretory proteins or components of unique or unusual signaling pathways.

A fourth area is to extend the use of murine models of aging, Sjogren's syndrome, and other lacrimal gland diseases to determine the molecular components altered by the disease

process. For example, in aging it would be advantageous to determine the molecular targets altered by invading mast cells and lymphocytes and the increase in oxidative stress.

A final area is to determine the effect of lacrimal gland secretory proteins on the function of the ocular surface. For example, a variety of growth factors are secreted by the lacrimal gland especially those in the EGF family. The effect of tear growth factors compared to endogenous ocular surface epithelial growth factors on ocular surface function is unknown. As another example, the secretory mucin MUC7 is elaborated by the lacrimal gland, but its effect on the ocular surface or in the tears is not yet known. A number of lacrimal gland proteins are antibacterial. It would be important to determine if there are additional antibacterial proteins in tears and their role in protecting the ocular surface from infection.

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Abbreviations

AD	autosomal dominant
ADVIRC	autosomal dominant vitreoretinopathopathy
AFVD	adult-onset foveomacular vitelliform dystrophy
AMD	age-related macular degeneration
ARB	autosomal recessive bestrophinopathy
BVMD	Best vitelliform macular dystrophy
CaV	voltage-dependent calcium
CERES	composite exonic regulatory element of splicing
CNV	choroidal neovascularization
EOG	electro-oculography
ERG	electroretinography
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
FAF	fundus autofluorescence
MRCS	microcornea, rod-cone dystrophy, early-onset cataract, and posterior staphyloma
OCT	optical coherence tomography
RPE	retinal pigment epithelium

Figure 1. Lacrimal Gland Functional Unit

Schematic of the components of the lacrimal gland functional unit that includes: (1) Activation of afferent sensory nerves from the cornea and conjunctiva that project through the central nervous system (CNS) to stimulate (2) Efferent parasympathetic and sympathetic nerves that innervate the lacrimal gland acinar and ductal cells and induce (3) Secretion of lacrimal gland fluid containing proteins, electrolytes, and water through the duct system onto the ocular surface that (4) drains into the lacrimal gland into the lacrimal drainage system. Modified from Rocha et al *The Ocular Surface* 6:162-174, 2008.

Figure 2. Schematic of the Structure of the Lacrimal Gland

This schematic shows the pyramidally shaped acinar cells linked at the luminal side by tight junctions that generate polarized secretion of proteins, electrolytes and water.

Parasympathetic and sympathetic nerves release neurotransmitters to activate their receptors on the basolateral membranes. Stimulation of these receptors induces a series of second messenger components that activate: (1) ion channels and pumps differentially located on the basolateral and apical membranes neurotransmitters to cause electrolyte and water secretion and (2) protein secretion by exocytosis. Ach-acetylcholine, M₃AChR-type 3 muscarinic acetylcholine receptor, VIP-vasoactive intestinal peptide, VIPR1-VIP receptor type 1, α_{1D}-AR- α adrenergic receptor type 1D, EGF- epidermal growth factor, EGFR-epidermal growth factor receptor

Figure 3. Immunofluorescence micrograph of lacrimal gland myoepithelial cells
Myoepithelial cells of rat lacrimal gland stained with an antibody to α smooth muscle actin. Myoepithelial cells are stellate-shaped cells that surround the basal aspect of acinar and ductal cells that are below the plane of focus.

Figure 4. Schematic of the functional types of sensory neurons innervating the ocular surface
 Four types of neurons are described with their differential location, basal activity, stimulated activity, and receptor type. Reprinted with permission from Belmonte et al The Ocular Surface 2:248, 2004.

Figure 5. Immunofluorescence micrographs of the distribution of parasympathetic, sympathetic, and sensory nerves in the lacrimal gland

Mouse lacrimal gland incubated with antibodies against various neurotransmitters. Anti-VIP demonstrates location of parasympathetic nerves surrounding most acini. Anti-tyrosine hydroxylase (TH) indicates sympathetic nerves that surround a few acinar. Anticalcitonin gene-related peptide (CGRP) shows sensory nerves with a limited distribution.

Figure 6. Overview of neural signaling pathways in the lacrimal gland

Stimulation of parasympathetic and sympathetic nerves releases their neurotransmitters to activate their receptors on the basolateral membranes. Stimulation of these receptors induces a series of stimulatory and inhibitory second messenger components that are specific for each neurotransmitter and induce protein secretion by exocytosis. Ach-acetylcholine, M₃AChR- type 3 muscarinic acetylcholine receptor, VIP-vasoactive intestinal peptide, VIPACR1- VIP receptor type 1, α_{1D}-AR- α adrenergic receptor type 1D, EGF- epidermal growth factor, EGFR-epidermal growth factor receptor, PLC-phospholipase C, PLD-phospholipase D, PKC- protein kinase C, MAPK-p44/p42 mitogen-activated protein kinase, NO-nitric oxide.

Figure 7. Schematic representation of parasympathetic signaling pathway

Acetylcholine (ACh) is released from parasympathetic nerves and binds to M₃ muscarinic receptor (M₃AChR). This initiates a signaling cascade that involves activation of phospholipase Cβ (PLCβ) and protein kinase C (PKC)α, ε, and δ, and the formation of diacylglycerol (DAG) and 1,4,5-inositol trisphosphate (IP₃). Calcium is released from the endoplasmic reticulum (ER) leading to protein secretion. The M₃AChR also activates non-receptor tyrosine kinases Pyk2 and Src to activate Ras, Raf, mitogen activated protein kinase (MEK) and p44/p42 mitogen-activated protein kinase (MAPK) that attenuates protein secretion.

Figure 8. Schematic representation of molecular mechanism of capacitative calcium entry
 Agonist activation of a plasma membrane receptor results in formation of inositol-1,4,5-trisphosphate (IP₃), which activates the IP₃ receptor causing discharge of store Ca²⁺ from a subcompartment of the endoplasmic reticulum. Within this subcompartment, Ca²⁺ binds reversibly to an EF hand motif in Stim1; depletion of Ca²⁺ results in Stim1 without Ca²⁺ bound, which causes Stim1 to redistribute within the endoplasmic reticulum to areas near Orai1 within the plasma membrane. Stim1 then activates Ca²⁺-selective Orai channels; the mechanism whereby this activation is accomplished is unknown. TRPC are additional mechanism for Ca²⁺ entry as they are non-selective cation channels that allow Ca²⁺ entry. Ag- agonist, G- guanine nucleotide-binding protein, PLC-phospholipase C, TRPC- transient receptor potential channel. Reprinted with permission from Putney Cell Calcium 42(2): 103-110, 2007.

Figure 9. Schematic Representation of VIP signaling pathway

VIP released from parasympathetic nerves binds to its receptors (VIPACR1 and II) and activates adenylyl cyclase (AC) to produce increase cellular levels of cAMP. cAMP activates protein kinase A (PKA) that stimulates protein secretion. Gαs- stimulatory guanine nucleotide-binding protein α subunit.

Figure 10. Schematic representation of the different components of A-kinase anchoring proteins (AKAPs)

There are numerous types of AKAPs that each bind a distinct set of signaling molecules in addition to protein kinase A (PKA). The Gravin AKAP binds phosphodiesterase. The muscle (m)AKAP binds phosphodiesterase and EPAC1 (exchange protein activated by cAMP) and extracellular regulated kinase (ERK, a mitogen-activated protein kinase). AKAP79 binds adenylyl cyclase. R-regulatory subunit of PKA, C- catalytic subunit of PKA. PDE- cAMP-dependent phosphodiesterase. Reprinted with permission from Beene and Scott, *Curr Opin Cell Biol* 19(2), 192-198. 2007.

Figure 11. Schematic of α_{1D} -adrenergic signaling pathway

Norepinephrine is released from sympathetic nerves to bind to the α_{1D} -adrenergic receptors (α_{1D} -ARs) that activates protein kinase C (PKC) ϵ and endothelial nitric oxide synthase (eNOS) to stimulate lacrimal gland protein secretion. α_{1D} -ARs also activate PKC α and - δ to attenuate protein secretion. In addition, matrix metalloproteinases (MMP) are activated by these receptors to transactivate the EGF receptor activating the Ras signaling cascade that also attenuates protein secretion. G α_q - guanine nucleotide-binding protein; NO – nitric oxide; GC – guanylate cyclase; Raf – mitogen activated protein kinase kinase; MEK – mitogen activated protein kinase kinase; MAPK – mitogen activated protein kinase.

Figure 12. Schematic of the Mechanism of Interaction of Ca^{2+} /protein kinase C and cAMP-dependent signaling pathways

The muscarinic, cholinergic Ca^{2+} /protein kinase C-dependent signaling pathway is illustrated here as an example of the EGF and α_{1D} -adrenergic signaling pathways. VIP stimulated increase in cAMP is illustrated as an example of mechanisms that increase cAMP. VIP released from parasympathetic nerves binds to its receptors (VIPACR1 and 2) and activates adenylyl cyclase (AC) to increase cellular levels of cAMP. cAMP activates protein kinase A (PKA) that inhibits activation of p44/p42 mitogen-activated protein kinase (MAPK) by cholinergic agonists. Cholinergic agonists, through increasing the intracellular $[\text{Ca}^{2+}]$ and activate protein kinase C (PKC), activate the non-receptor tyrosine kinases Pyk2 and Src to activate MAPK. G α s-stimulatory guanine nucleotide-binding protein, PDE-cAMP-dependent phosphodiesterase, Ras, Raf-mitogen-activated protein kinase kinase, MEK-mitogen-activated protein kinase kinase.

Figure 13. Schematic of working model for mechanism of basal and stimulated exocytosis for protein secretion

(A) Resting lacrimal gland acini maintain mature secretory vesicles (mSVs) enriched in Rab3D beneath dense apical filament network. The mSVs utilize cytoplasmic dynein for their maturation and to maintain their subapical localization. Other recruitable SVs (rSVs) enriched in VAMP2 are present in the cytosol. (B) Upon agonist stimulation there is an immediate thinning of apical actin filaments concomitant with the loss of Rab3D from primed individual SVs in clusters accompanying assembly of actin and non-muscle myosin II filaments around these clusters. Contractile forces generated by the acto-myosin system results in compound fusion of individual SVs and movement of these fusion intermediates toward the apical plasma membrane (APM). Ultimately, they reach the APM where they are able to gain access to appropriate SNARE proteins and complete the fusion step. At the same time, rSVs in the cytosol move along the microtubules (MTs) with the aid of cytoplasmic dynein to the APM, where they fuse and release their contents through formation of a SNARE pair with rSV VAMP2 and the APM syntaxin 3. Reprinted with permission from Wu et al *Experimental Eye Research* 83:84-96, 2006.

Figure 14. Schematic of transcytosis mechanism of protein secretion

Secretion of dimeric IgA (dIgA) illustrates the transcytosis secretory mechanism. Polymeric IgA receptor (pIgR) trafficks dIgA leading to secretion of secretory IgA and secretory component (SC). pIgR and SC can be secreted by exocytosis via the process described in Figure 13 and shown with black arrows. These compounds can also be secreted by transcytosis by the process illustrated in red arrows. ER-endoplasmic reticulum, BE-basolateral endosomes, TGN-transgolgi network, AE- apical endosomes, SV- secretory vesicles. Reprinted with permission from Evans et al Am J Physiology Cell Physiol 294:C662-C674, 2008.

Figure 15. Mechanism of electrolyte and water secretion by lacrimal gland acinar cells
 NKA, Na⁺,K⁺-ATPase, AE, anion (Cl⁻/HCO₃⁻) exchanger, NHE, Na⁺/H⁺ exchanger, NKCC- Na⁺-K⁺-2Cl⁻ cotransporter. Reprinted with permission from Selvam et al Am J Cell Physiol 293:C1412-C1419, 2007.

Figure 16. Model for secretion of electrolytes and water by lacrimal gland duct cells
 Parasympathetic agonists increase the intracellular $[Ca^{2+}]$ that stimulates the Na^+/H^+ exchanger (NHE) followed by activation of the anion (Cl^-/HCO_3^-) exchanger (AE) on the basolateral membrane that drives Na^+ and Cl^- into the duct cells. The Na^+ and Cl^- is dependent upon the generation of H^+ and HCO_3^- generated by intracellular carbonic anhydrase (CA). The elevated Na^+ can be exchanged for K^+ through the basolateral Na^+, K^+ -ATPase, which increases cellular K^+ concentration. The increase in intracellular K^+ and Cl^- drives these ions out of the cell into the lumen via specific K^+ and Cl^- channels (IK_{Ca1} and $CIC3$, respectively) and a K^+/Cl^- co-transporter. An increase in intracellular $[Ca^{2+}]$ can also stimulate IK_{Ca1} . Reprinted with permission from Toth-Molnar et al Invest Ophthalmol Vis Sci 48(8);3746-3755,2007.

Figure 17. Schematic Representation of the neural regulation of lacrimal gland secretion in the diseased state

Proinflammatory cytokines block the release of neurotransmitters from the afferent sensory nerves and the efferent parasympathetic and sympathetic nerves preventing their stimulation of lacrimal gland fluid secretion resulting in an inadequate secretion leading to aqueous deficiency dry eye. Modified from Dartt et al *The Ocular Surface* 2:76-91, 2004.