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Adrenergic and possibly nitric oxide-ergic modulation of mouse lacrimal gland function.

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ADRENERGIC AND POSSIBLY NO-ERGIC MODULATION OF MOUSE LACRIMAL GLAND FUNCTION

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT OF DISSERTATION
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PURPOSE. In the currently accepted model of lacrimal gland function, the parasympathetic nervous system is thought to be responsible for most of the secretory functions. The adrenergic system has been assumed to play an indirect and minor role. However, there is evidence that the adrenergic system may directly influence tear secretion from the gland. Several lines of evidence also suggest that nitric oxide may play a role in modulating tear secretion. The purposes of the dissertation are to investigate the adrenergic innervation in the mouse lacrimal gland and its functional relevance to protein secretion. We have also studied the possible expression of neuronal nitric oxide synthase (nNOS) in the gland.

METHODS. Mouse lacrimal gland and pterygopalatine ganglion (PPG) were processed for single- and double-labeled indirect immunofluorescence studies and examined with conventional and confocal microscopy. Sucrose-potassium phosphate-glyoxylic acid (SPG) method was also used to visualize the adrenergic system. Protein secretion and SDS-PAGE experiments were performed to study the lacrimal glands' responses to agonists and their corresponding protein profiles.

RESULTS. The mouse lacrimal gland can be divided into two different areas based on the innervation density and distribution pattern. Adrenergic agonists, phenylephrine and norepinephrine, induced a similar magnitude of protein secretion to

that of the cholinergic agonist at concentrations from 10^{-6} to 10^{-4} M. Extensive α_1 and β_1 adrenergic receptor immunoreactivity was observed in the gland. Our data showed no synergism between α_1 and β receptor-stimulated protein secretion. Preliminary SDS-PAGE data suggest there are differences between the proteins secreted in response to α_1 and β adrenergic stimulation, which supports the notion of differential secretion in the mouse lacrimal gland. nNOS immunoreactivity was observed in the neurons in the PPG and nerve fibers in the lacrimal gland and colocalized with both parasympathetic and adrenergic nerves.

DISCUSSIONS. These data suggest that there is much more adrenergic innervation in the mouse lacrimal gland than previously thought, and that it has a direct effect on protein secretion. Both immunofluorescence and functional studies suggest the presence of α_1 and β_1 receptor-mediated protein secretion pathways in the mouse lacrimal gland and it appeared that the adrenergic activation-induced protein secretion was mainly mediated by α receptors. The observation of nNOS immunoreactivity in the PPG and nerve fibers in the lacrimal gland suggest that nitric oxide may play a role in modulating tear production.

DEDICATION

To my wife, Mingxia, for her unwavering love.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AR	Adrenergic Receptor
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanosine Monophosphate
DAG	Diacylglycerol
IgA	Immunoglobulin A
IR	Immunoreactivity
NO	Nitric Oxide
nNOS	Neuronal Nitric Oxide Synthase
PPG	Pterygopalatine Ganglion
sIgA	Secretory Immunoglobulin A

INTRODUCTION

Gross Anatomy and Histology of the Mammalian Lacrimal Gland

Adequate production of tears of the appropriate composition is essential to maintain the health of the ocular surface. Most of the tears that bathe the eyes in mammals come from the major exorbital lacrimal glands, which are situated within the orbit in mammals such as rabbits. In contrast, in rodents, such as rats and mice, the gland is outside the orbit and on the lateral side of the face near the ear, under the skin.

A typical mammalian lacrimal gland consists of a number of lobes that are separated by interstitial tissue. These lobes are further subdivided into lobules, which are comprised of acini, the functional units of the gland. An acinus is formed by secretory acinar cells surrounding a central lumen. The acinar cells are columnar in shape and are connected by extensive junctional complexes, including tight junctions and gap junctions, in the apical portion. Tight junctions are the basis of the blood-tear barrier, preventing molecules in the interstitial areas from diffusing freely into the lumen. Gap junctions serve as channels that electrically couple the acinar cells. Small molecules, including some second messengers, may also diffuse through gap junctions to spread signals from one cell to others upon stimulation.

The apical surfaces of acinar cells have many microvilli that project into the lumen, whereas the lateral membranes are often folded to increase their surface area, facilitating the transport of secretory products. Like other secretory epithelia, the lacrimal acinar cells are filled with large numbers of secretory vesicles in the apical portion, with

nuclei situated in the basal portion and a large perinuclear Golgi apparatus, where the modification of secreted proteins takes place.

The acinar cells are responsible for most of the secretory products, including proteins, ions, and water. The secretory product of the acini, the primary tear fluid, is collected by the intra-acinar lumen and then drains to the intra-lobular and inter-lobular ducts, and finally to the main collecting ducts that convey the tears to the eye.

The ductal cells are similar to acinar cells, except that they are more cuboidal in shape. These cells are responsible for the modification of primary tears and their transport.

In the interstitial areas of mammalian lacrimal glands, some accessory cell types are also present, such as myoepithelial cells, lymphocytes/plasma cells, mast cells, and fibroblasts. Myoepithelial cells are often observed to be attached to acinar and ductal cells in a lacy arrangement, and they contain a network of α-smooth muscle actin that is similar to that of smooth muscle.[1,2] M_3 muscarinic receptors are expressed on their surfaces,[2] and for this reason these cells have been thought to be involved in the release of secretory product by contraction. Other investigators have proposed that the myoepithelial cells serve only as the "exoskeleton" of the acini.[3]

Normally, both B and T type lymphocytes, as well as plasma cells, are present in the interstitial areas of the gland. Most of the plasma cells are responsible for the secretion of immunoglobulin A (IgA) in the tears. Following secretion into the interstitium, IgA is then actively transported across the acinar cells into the lumen to mix with other components of the tears, which finally drain onto the surface of the eye.

Fibroblasts and mast cells are less commonly observed in the gland. Fibroblasts produce the collagen fibers and matrix that are observed to fill the interstitial space, whereas mast cells secrete both histamine and heparin. The interstitial areas are also filled with collagens, extensive blood vessels, nerve bundles, and fibers.

Autonomic Innervation of the Lacrimal Gland

The tear secretion from the exorbital lacrimal gland is highly regulated and is controlled by a number of factors, both internal and external. Under normal physiological conditions, the tear flow rate is less than 1 μL/min, whereas the maximal rate can exceed 100 μL/min in the stimulated state.[4] Therefore, the secretion and release processes must be highly regulated by various systems, including the nervous system.

The mammalian lacrimal gland is innervated by the autonomic nervous system, with the parasympathetic system predominating.[5-10] However, some sympathetic innervation has been reported in some species,[11-14] as well as nerve fibers that contain neuropeptides such as vasoactive intestinal polypeptide (VIP), substance P (SP), Leu-enkephaline (L-Enk), neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP). The available data suggest that, in most species, the innervation of the gland includes both classical neurotransmitters such as acetylcholine (ACh) and norepinephrine, as well as at least two neuropeptides. In addition to the complex neurotransmitter systems in the gland, there is also evidence that some neuropeptides co-exist with classical transmitters. VIP has been found to coexist and to be coreleased with acetylcholine in submandibular glands.[15-18] Although there is no direct evidence that VIP coexists and is coreleased with

ACh in the lacrimal gland, the similarity between these two glands suggests that this may be the case.[19]

In addition to the conventional autonomic neural supply to the gland, there is increasing data suggesting that the lacrimal gland secretions, including ions, water, and protein, may be also influenced by unconventional neurotransmitters, such as nitric oxide (NO). NO has also been demonstrated to modulate the release and effects of other neurotransmitters as discussed below.

The functional significance of the complex innervation pattern with multiple neurotransmitters and modulators in the mammalian lacrimal gland is still unclear. Most neurotransmitters and peptides, including ACh, norepinephrine, and VIP, can increase protein and fluid secretion. The only transmitters reported to have an inhibitory effect on lacrimal secretion are members of the enkephalin family. Two hypotheses have been proposed to explain the complex innervation of the lacrimal gland. One view is that the complex innervation pattern represents "redundancy," a safety factor, in case of failure of one or more of the innervation pathways. Other exocrine glands, like the salivary and adrenal gland, also have similarly complex innervation patterns. Another view is that different mixes of neurotransmitters (both classical and unconventional) and neuromodulators will produce tear fluid of different composition in different conditions to cater to different needs. Some reports appear to support this idea.[20] Our observations indicate that there are regional differences in the innervation density and distribution pattern in the mouse lacrimal gland, which may lend support to the idea that there are different populations of secretory cells in the lacrimal gland and that the selective activation of specific subsets may produce tear fluid of differing composition.

Both immunohistochemical and ultrastructural studies have shown that each acinus in the gland is innervated. However, it is also clear that not every acinar cell is in association with a nerve fiber. It has been proposed that the extensive gap junctions among acinar cells, as shown by both ultrastructural and dye injection studies, are responsible for the spread of neural signals upon stimulation.

Nerve fibers are observed to be in close association with the basolateral side of both acinar and ductal cells. Various neurotransmitter/modulator receptors have been shown to reside in the basolateral membrane of these cells.[21] Neurotransmitters or modulators released from nerve terminals, or that diffuse from the blood stream in the adjacent capillaries (like norepinephrine secreted by adrenal gland), bind to their corresponding receptors. Agonist binding to these receptors will activate the corresponding intracelluar transduction pathways and tear fluid secretion.

Parasympathetic innervation. The parasympathetic nerve fibers in the lacrimal gland arise from neurons in the pterygopalatine ganglion (PPG).[22-26] The PPG is the parasympathetic ganglion that receives synaptic input from preganglionic parasympathetic neurons in the brainstem. Postganglionic nerve fibers from the PPG innervate the lacrimal gland, the nasal and palatine mucosae, and the extra- and intracranial vasculature.[26-27]

Microscopic analysis at both the light and electron microscopy level using protocols that reveal the distribution of acetylcholinesterase (AChE), choline acetyltransferase (ChAT), or vesicular acetylcholine transporter (VAChT) have shown that each acinus of the mammalian lacrimal gland is directly innervated by

parasymathetic nerves.[21] ChAT and AChE are the enzymes that are responsible for the synthesis and breakdown, respectively, of ACh.[28] VAChT is a proton-dependent transporter that is responsible for packaging ACh into synaptic vesicles.[29-32] Both *in vivo* and *in vitro* studies have also shown that ACh or cholinergic agonists like carbachol can induce protein, electrolyte, and water secretion. Therefore, the parasympathetic system has long been assumed to play a major role in modulating tear production.

The ACh receptors on the lacrimal gland are of the muscarinic type (mAChR). Of the five subtypes of mAChR, M_3 has been shown to be present in the lacrimal gland.[33,34] Binding of ACh to mAChRs will activate G proteins, and intracellular signal transduction is activated subsequently through the Ca^{2+}/diacylglycerol (DAG)/cGMP-dependent pathway.

Sympathetic innervation. The sympathetic nerves within the lacrimal gland originate primarily from the superior cervical ganglion (SCG), which is the uppermost ganglion of the sympathetic trunk that lies in the upper part of the neck.[35]

The sympathetic system has long been assumed to have only a supplemental role in lacrimal gland function by controlling blood flow to the gland and blood distribution within it, or to have no contribution at all in some species.[36-39] However, there is increasing evidence that the sympathetic nervous system plays a role not only in modulating blood flow within the gland, but that it may also have direct effects on tear secretion in some species.[20,40,41] Other investigators have shown that the sympathetic innervation of the mammalian lacrimal gland is highly variable among species, although there are controversies in the literature.[12,14,42] For example, there are reports that every

acinar cell is in close proximity to an adrenergic fiber in cat,[11] dog,[12,14] human, and monkey.[13] However, there are other studies in humans[43] and in monkeys[44] that are inconsistent with these findings. In rat[12,42] and mouse[12], only very sparse tyrosine hydroxylase (TH)-positive nerve fibers have been described and most of them were in association with the blood vessels, while only a few were found among acini. In guinea pigs, some investigators described rich adrenergic innervation in lacrimal glands,[42] while others reported a virtual absence in the gland, except along the blood vessels.[12]

The neurotransmitter released by adrenergic nerves is norepinephrine, which activates both α and β adrenergic receptors. Adrenergic receptors are currently separated into three families: α_1, α_2, and β. Each of them is subdivided into three subclasses. Different adrenergic receptor subtypes bind to G proteins that in turn can alter the activity of phospholipase C, modulate Ca^{2+} channels, and activate adenylyl cyclase. The activation of G proteins and intracellular transduction pathways result in protein, ion, and water secretions.

NO and its possible role in lacrimal gland secretion. Nitric oxide is produced by nerve terminals, vascular endothelium, and inflammatory cells in a reaction catalyzed by nitric oxide synthase (NOS), which converts L-arginine to NO and L-citrulline.[45] There are at least three isoforms of NOS, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS).[46,47] Both nNOS and eNOS are constitutively expressed, but their activity is regulated by various soluble ligands and/or shear forces through calcium /calmodulin-dependent second messenger pathways. NO concentrations produced by this route are usually relatively low (in the picomolar range). In contrast, regulation of iNOS

occurs at the gene transcription level and is relatively slow, but leads to long-lasting and significant increases in NO production (in nanomolar range local concentration).[45,47,48]

NO mediates a variety of physiological functions in both central and peripheral nervous systems.[49-60] NO is a small, lipid-soluble, and highly diffusible compound, which easily penetrates the membrane of nearby cells and can therefore affect them in a receptor-independent autocrine and/or paracrine manner. However, NO has a short half-life and is rapidly converted to nitrites and nitrates, hence its range of action is limited. At the same time, NO is a reactive free radical, due to an unpaired electron in its molecular orbital. At low concentrations, it preferentially activates guanylate cyclase (GC), which produces cGMP. At high concentrations, NO inhibits various iron-containing DNA synthases and mitochondrial enzymes, and thus inhibits cell growth and division, leading ultimately to cell death.[61]

NO is eliminated by oxyhemoglobin under normal physiological conditions. However, if NO is overproduced, it will combine with another free radical, superoxide ($O_2\cdot^-$), forming another free radical, peroxynitrite ($ONOO^-$), which is highly active and can cause tissue damage. Peroxynitrite preferentially interacts with tyrosine, forming nitrotyrosine, which is the hallmark of nitration and has been used to detect tissue damage induced by NO.

The various physiological and pathological functions that NO mediates in the central and peripheral nervous systems are well documented, and its role in regulating cranial exocrine gland secretion, such as that of the salivary glands, has been described.

Immunohistochemical studies demonstrated that NOS was found in several cranial exocrine glands, such as the submandibular glands of rat,[62-64] cat ,[65] and ferret,[64]

the labial glands of humans,[66] and the parotid and sublingual glands of rat and ferret.[64] Previous studies have shown that nNOS-IR is expressed in the PPG[67-69] and nasal mucosae of rats,[70] and humans.[71]

Physiological and biochemical studies in salivary glands (the submandibular and parotid glands), have shown that NO plays a role in modulating saliva secretion. In the rat, cat, and ferret, NO has been implicated in salivary secretion from the submandibular gland.[62,72-75] NO has also been demonstrated to increase protein and electrolyte production in the parotid gland of sheep.[76]

To our knowledge, there are no published reports concerning a potential role for NO in modulating tear production in lacrimal glands. Anatomically and functionally, the lacrimal gland is similar to the salivary glands. Salivary glands also receive dense parasympathetic innervation from the PPG, which is the parasympathetic source for other tissues including the nasal mucosae in rats[77] and humans.[71] We hypothesize that nNOS exists in the neurons that innervate the mouse lacrimal gland and neural cell bodies in the ganglia from which parasympathetic nerves in the gland originate, and that NO may play a role in modulating tear production.

In agreement with immunohistochemical, physiological, and biochemical studies of other cranial exocrine glands, our preliminary immunohistochemical results have indicated that nNOS-IR was observed in association with nerve fibers in the mouse exorbital lacrimal gland.[78-81] nNOS-IR was widely expressed in nerve fibers that were in close proximity with acini, ducts, and blood vessels in the mouse lacrimal gland, suggesting that NO might play a role in modulating tear production in the lacrimal gland.[82]

The extent and mechanism of NO's modulatory effects on tear production are unclear. However, several lines of evidence suggest that NO may exert its effect through its interaction with other neurotransmitter/modulators.[83-85] VIP has been found to be co-localized and co-released with ACh in postganglionic, parasympathetic nerve terminals in the submandibular glands.[15,17,18] In the submandibular glands of the cat and ferret, NO has been reported to increase protein secretion by mediating VIP release.[72-74] Reports on other tissues demonstrate that NO is also implicated in the presynaptic VIP release in guinea pig stomach and rat colon *in vitro*.[86,87] Studies in *Torpedo* synaptosomes have indicated that NO may decrease ACh release,[88] and other reports have shown that NO can act to modulate synaptic transmission at several types of synapses.[88-92] VIP has been demonstrated to stimulate protein secretion in the rat lacrimal gland.[19] Although there is no direct evidence as yet for the co-localization and co-release of VIP and ACh in the lacrimal gland, available data suggest that this might be the case.

NO may also influence tear secretion through various mechanisms, including control of cGMP production,[93-96] modulation of ion channels,[97,98] activation of intracellular signaling pathways,[99] and regulation of Na^+, K^+-ATPase.[100] Several other mechanisms have also been proposed.[101,102]

Innervation density and distribution pattern. The available data indicate that the innervation distribution in the mammalian lacrimal gland is relatively even,[42,103,104] although innervation in the rat has been reported to be most abundant in the hilus,[21] where the main collecting ducts exit the gland.

In contrast to the mammalian lacrimal gland, the avian Harderian gland, which is the major lacrimal gland in birds, has two anatomically distinct areas, the cortex and medulla, and these areas display different innervation patterns. The cortex consists of secretory tubules made of columnar epithelium, whereas the medulla contains only a few. Both parasympathetic and adrenergic nerves are found throughout the Harderian gland, but only sparse innervation is observed around the cortical secretory acini in pigeons[105] and chickens,[21,106] in contrast to the dense innervation observed in the medulla. However, the mouse lacrimal gland is similar to that of other mammals in that it is not comprised of two anatomically distinct areas.

Signal Transduction Pathways of the Lacrimal Gland

Two separate intracellular transduction systems have been proposed, designated by the second messengers they use: 1) Ca^{2+}/DAG/cGMP-dependent pathway; and 2) cAMP-dependent pathway.[1,19,107]

The Ca^{2+}/DAG/cGMP-dependent pathway. The transmitter first binds to the receptors and activates a guanine nucleotide-binding protein (G protein) in the plasma membrane. Activation of this G protein stimulates phosphatidylinositol bisphosphate (PIP_2) phosphodiesterase (phospholipase C) to break down PIP_2 into 1,4,5-inositol trisphosphate (1,4,5-IP_3) and DAG. IP_3 can release Ca^{2+} from intracellular stores. The increase of intracellular Ca^{2+} concentration, in conjunction with calmodulin, activates Ca^{2+}/calmodulin-dependent protein kinases to phosphorylate and activate specific protein substrates, which can induce secretion directly. DAG can translocate protein kinase C

(PKC) from the cytosol to the apical membrane. At the plasma membrane, activated PKC phosphorylates protein substrates. These phosphorylated proteins are assumed to play a direct role in inducing secretion.

The receptor systems that trigger this signaling cascade include the muscarinic acetylcholine receptors and partially α_1-adrenergic receptor activation.

The cAMP-dependent pathway. Stimuli that activate the cAMP-dependent pathway bind to their receptors in the plasma membrane and activate G protein, either stimulatory (Gs) or inhibitory (Gi). Gs activation can increase adenylyl cyclase (AC) activity to produce cAMP, the second messenger, from adenosine triphosphate (ATP). The increase in cAMP concentration stimulates cAMP-dependent protein kinase A (PKA) to phosphorylate and activate its protein substrates. These phosphorylated proteins are thought to induce secretion directly. In contrast, activation of Gi causes it to bind to AC, preventing its activation by Gs and therefore blocking the increase in intracellular cAMP levels.

Agonists that use this pathway to stimulate secretion include VIP and, to a limited extent, β-adrenergic agonists. Members of the enkephalin family have been identified to be the only inhibitors of this pathway.

Protein Secretion of the Lacrimal Gland.

Protein secretion by the mammalian lacrimal gland is primarily accomplished by the acinar cells. Acinar cells are connected with each other by tight junctions in the luminar side, which is the basis of the blood-tear barrier that prevents macromolecules

from entering the duct system through the intercellular space. The acinar cell plasma membrane can be divided into basolateral and apical sides separated by tight junctions. Neurotransmitter receptors are found on the basolateral side membrane and are surrounded by nerve endings.

There are two kinds of protein secretion: regulated and constitutive secretion. Regulated protein secretion occurs in response to an increase in intracellular second messengers. Secretory proteins are synthesized in the endoplasmic reticulum and modified in the Golgi apparatus. The proteins are then packaged into secretory granules, which are stored near the apical membrane side. Upon appropriate stimulation, such as activation of the receptors on the acinar cells, intracellular transduction systems are activated, second messenger levels are increased, the secretory granules fuse with the apical membrane, and the proteins are exocytosed into the lumen. This regulated protein secretion is controlled by the level of intracellular second messengers. The regulated protein secretion rate increases with increasing fluid secretion, namely, its concentration in the fluid is virtually unaffected by stimulation. Some of the proteins secreted by the lacrimal gland are lactoferrin, lipocalin (tear specific prealbumin), lysozyme, peroxidase, amylase, plasminogen activator, prolactin, epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and endothelin-1.

Constitutive proteins are secreted by a constitutive pathway, and secretion in this instance is controlled by the rate of protein synthesis. These proteins are synthesized in the same organelles as regulated proteins, but they are packaged into different vesicles in the Golgi apparatus. The vesicles of constitutive proteins are larger and more electronlucent than vesicles of regulated proteins, and they have a shorter half-life. The

release mechanism is the same as that of regulated proteins. The constitutive protein secretion rate depends on the fluid secretion rate, and the rate decreases with increasing fluid secretion. Secretory immunoglobulin A (sIgA) is the major constitutive secretory protein in mammalian lacrimal gland.

Unlike regulated proteins, which arise from the acinar and ductal cells, IgA is synthesized by plasma cells scattered in the interstitial areas. The IgA is initially secreted by plasma cells as a dimer, linked by a protein called "*J* chain" which is also produced by plasma cells. The complex is called sIgA. The sIgA binds to secretory component (SC), a glycoprotein produced by acinar cells that serves to transport through them, on the baso-lateral membrane of the acinar cells. The SC-sIgA complex is then endocytosed in vesicles and transported across the acinar cells to reach the apical membrane. These vesicles fuse with the apical membranes, and the SC is cleaved into two portions, one of which is recycled by the acinar cells while the other remains attached to the sIgA complex.

Electrolytes, Water Secretion and Tear Fluid Modification

Electrolyte and water are the major secretory products of the lacrimal gland.[108,109] Tear fluid can be considered to be produced in two stages: primary tears are secreted into the lumen by acinar cells and then modified by ductal cells in the duct system.

The apical membrane of acinar cells contains Cl^- and K^+ channels to allow the movement of solutes across the membrane. Aquaporin 5, the water channel, is also believed to be present in the apical membrane to facilitate water movement across the epithelium. On the basolateral membranes, large amounts of Na^+-K^+-ATPase are present,

along with K^+, Cl^-, and Ca^{2+} channels, as well as less specific cation and anion channels. The Na^+-K^+-ATPase actively moves K^+ into the cell and Na^+ out the cell, generating and maintaining the Na^+-K^+ gradients, which provide the driving force for the movement of electrolytes and water across the epithelium. Several other coupled transport systems (porters), which are driven by the concentration gradients generated by the Na^+-K^+-ATPase and carbonic anhydrase, are also present in the basolateral side of the acinar cells. Water passively diffuses into the lumen from interstitial spaces due to osmotic pressure established by the movement of electrolytes.

Although little is known about the transport physiology of the lacrimal duct system, it has been estimated that the ducts account for about 30% of the final lacrimal gland fluid volume. The primary tear fluid, while being transported through the ductal system, is modified by the ductal cells. Several kinds of channels are assumed to be present in the ductal cells, K^+ and Cl^- channels, K^+-Cl^- cotransporters, and K^+-H^+ antiporters.

Research Topics

Based on previous data and our preliminary findings, we proposed the following hypotheses: 1) the mouse lacrimal gland receives more extensive adrenergic innervation than previously realized, 2) the adrenergic input may play a direct and important role in modulating or controlling protein production and secretion from the mouse lacrimal gland, and 3) there are regional differences of innervation density in the mouse lacrimal gland.

The results of our studies that were undertaken to test these hypotheses are discussed in three papers.

The first paper addresses the regional differences of innervation density and distribution pattern, and the sympathetic neural control of protein secretion in the mouse lacrimal gland. The results of studies that relied on both immunohistochemical and sucrose-potassium phosphate-glyoxylic acid (SPG) techniques showed that the mouse lacrimal gland can be divided into two different areas based on the innervation density and distribution pattern. The regional differences of innervation density appear to have some similarities to the innervation pattern in the avian Harderian gland. The avian Harderian gland showed highly uneven innervation distribution, as much more innervation was observed in the medulla than the cortex. Adrenergic agonists induced a similar magnitude of protein secretion to that of the cholinergic agonist. These data suggest that the sympathetic innervation of the mouse lacrimal gland is much more extensive than previously reported and is associated with the protein secretory processes. The division of the lacrimal gland into two areas suggests that the mouse lacrimal gland might be a mixed gland and that these two areas may play different roles in secreting tears of different composition in different situations. Our data appear to support the notion that differential secretion is accomplished by activating different populations of cells.

The second paper addresses the observations of α_1 and β_1 adrenergic receptor (AR) immunoreactivity in the mouse lacrimal gland, and protein secretion in response to the adrenergic agonists phenylephrine (α_1 AR agonist) and isoproterenol (β_1 AR agonist), with or without their corresponding antagonists (phentolamine for α AR and propranolol

for β AR). These data demonstrated that the direct stimulation of either α_1 or β_1 AR could induce significant protein secretion from the mouse lacrimal gland fragments. The specificity of this stimulation was further indicated by the antagonistic effects of adrenergic receptor antagonists. Our results appear to support the notion that there is extensive adrenergic control in the mouse gland. We propose that the adrenergic receptors may be better markers to reflect the extent of adrenergic control because circulating norepinephrine in the blood stream should be taken into consideration. Using other markers, like TH or dopamine β-hydroxylase, may underestimate the extent of adrenergic control, as they only reflect local sources of norepinephrine. Our immunohistochemical and biochemical findings support the notion that there is extensive α_1 and β_1 adrenergic receptor-mediated pathways in the mouse lacrimal gland and that this system plays a direct and significant role in protein secretion. Our data showed no synergism between α_1 and β receptor-mediated protein secretions. Preliminary data also suggest there are differences between proteins induced by α_1 and β adrenergic systems, which appear to support the notion of differential secretion in the mouse lacrimal gland.

The third paper addresses the immunohistochemical observations of nNOS immunoreactivity in neurons in the PPG and nerves in the lacrimal gland, by using both confocal and conventional microscopy techniques. The findings suggest that NO may play a role in modulating tear production. The site of action might include the PPG, ducts, blood vessels, acini, nerve fibers, and myoepithelial cells. NO may modulate parasympathetic and sympathetic synaptic transmission, or directly act on lacrimal gland components. The interaction between NO-ergic and the conventional autonomic input illustrates the complexity of the innervation pattern of the mouse lacrimal gland.

THE SYMPATHETIC NEURAL CONTROL OF THE MOUSE LACRIMAL GLAND

by

CHUANQING DING, BENJAMIN WALCOTT, AND KENT T. KEYSER

Submitted to *Investigative Ophthalmology & Visual Science*

Format adapted for dissertation

ABSTRACT

PURPOSE. To explore the sympathetic innervation pattern and the role of the sympathetic nervous system control in protein secretion in the exorbital lacrimal glands of normal mice.

METHODS. Mouse lacrimal glands were processed for single- and double-labeled indirect immunofluorescence studies to show their innervation patterns. The sucrose-potassium phosphate-glyoxylic acid method was also used to visualize the adrenergic innervation. The effects of adrenergic and cholinergic agonists on protein secretion were evaluated.

RESULTS. The mouse lacrimal gland can be divided into two different areas based on the innervation density and distribution pattern. One area, about 10~30% of the gland, exhibited much higher innervation density, both parasympathetic and sympathetic, than the rest of the gland. Adrenergic agonists, norepinephrine and phenylephrine, induced increases in protein secretion that were of similar magnitude to the increases induced by the cholinergic agonist, carbachol, at 10^{-6} to 10^{-4} M. Isoproterenol, the β adrenergic agonist, also elicited protein secretion at 10^{-5} to 10^{-4} M.

CONCLUSIONS. Our data indicate that there is extensive sympathetic innervation of the mouse lacrimal gland, and that sympathetic input can modulate protein secretion. The division of the lacrimal gland into two areas suggests that the mouse lacrimal gland is a mixed gland and that these two areas may play different roles in secreting tears of different compositions in different situations. Our data appeared to support the notion that differential secretion is accomplished by activating different populations of secretory cells that are differentially innervated.

INTRODUCTION

The adequate production of tears of the appropriate composition is essential in maintaining the clarity of the cornea. Most of the fluid and proteins that supply the mammalian eye are from the major lacrimal glands. In rodents, a major lacrimal gland is the exorbital lacrimal gland found near the ear on the lateral side of the skull. This gland is innervated by the autonomic nervous system, with parasympathetic input predominating,[1-3] although some sympathetic innervation has been reported in some species.[1-7] The innervation is reported to be evenly distributed,[8-10] although the innervation in rats has been reported to be most abundant in the hilus.[11]

While it is generally accepted that the parasympathetic innervation of the lacrimal gland is responsible for inducing most tear secretion, there is still some controversy over the functional relevance of the sympathetic innervation. There is evidence that the sympathetic nervous system plays a direct and significant role in initiating and modulating tear secretion in some species, instead of only modulating blood flow to the gland.[12,13] The sympathetic innervation varies greatly among species, and there are some discrepancies in the literature. For example, there are reports that every acinar cell is in close proximity to an adrenergic fiber in cats,[4] dogs,[5,7] humans, and monkeys.[6] However, other data from studies in humans[14] and monkeys[15] are inconsistent with these observations. In rats[5,10] and mice,[5,9,16] only sparse adrenergic nerve fibers have been reported, mostly in association with blood vessels, with only a few being found among acini. In guinea pigs, rich adrenergic innervation in lacrimal glands has been reported,[10] while others described a virtual absence in the gland except along the blood vessels.[5] To resolve these controversies and to determine the innervation pattern in the mouse lacrimal

gland, a useful system in the study of lacrimal gland function, we have used histochemical and immunohistochemical techniques to examine the sympathetic innervation pattern. We have also determined whether various α and β adrenergic agonists induced glandular protein secretion. We found that some lobes of the mouse lacrimal gland displayed a high density of tyrosine hydroxylase (TH) immunoreactive fibers, a marker for sympathetic fibers,[9] among the acini, while in other lobes the sympathetic innervation to the acini was sparse. Antibodies to the vesicular acetylcholine transporter (VAChT) and to the synaptophysin show that the cholinergic and total innervation is also not distributed evenly among the lobes of the gland. Both cholingergic and adrenergic agonists induce protein secretion by gland fragments, suggesting that both have a role in the regulation of gland function.

MATERIALS AND METHODS

Chemicals

Carbamylcholine chloride (carbachol), isoproterenol bitartrate, norepinephrine bitartrate, L-phenylephrine hydrochloride, phentolamine hydrochloride, and DL-propranolol hydrochloride were from Sigma (St. Louis, MO). All other reagents were of the highest purity available.

Animals

C57 female mice (~18 g body weight), aged from 2 to 12 months, were purchased from commercial vendors (Taconic Farms, Germantown, NY, or Charles River, Wilmington, MA). All animals were kept in a 12-hour light-dark cycle and maintained in

an accredited animal facility with freely available food and water. They were managed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To obtain lacrimal glands, the animals were sacrificed with an overdose of halothane around midday, and lacrimal glands were rapidly removed and trimmed from surrounding membranes and fatty tissues under a dissecting microscope.

Tissue Preparation for Immunohistochemistry

For immunohistochemistry, lacrimal glands were fixed in freshly prepared 4% buffered paraformaldehyde. After 3-4 hours of fixation at 4 °C, the tissue was placed in 0.1 M phosphate buffer containing 30% sucrose at pH 7.4 for at least 12 hours at 4 °C. The glands were then placed in optimal cutting temperature (OCT) embedding medium (Sakura Finetek USA, Torrance, CA), serially sectioned at 10 μm on a cryostat (Leica, Deerfield, IL), and collected on slides (SuperFrost Plus; VWR Scientific, West Chester, PA). The sections were dried and stored at –20 °C until used.

Immunohistochemistry

The antibodies used were sheep (dilution 1:800) and rabbit (dilution 1:400) anti-TH polyclonal antibodies (Chemicon International, Temecula, CA); rabbit anti-synaptophysin polyclonal antibody (Dako, Carpinteria, CA) at a dilution of 1:200; and goat anti-VAChT polyclonal antibody (Chemicon) at a dilution of 1:2,000. The VAChT antibody has been well characterized and is reported to colocalize with classical cholinergic markers, such as choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), in both the central and peripheral nervous systems, and in the lacrimal gland.[17,18]

Sections were incubated in primary antibody diluted with 0.1 M sodium phosphate buffer (PBS) overnight or for 48 hours for TH, at 4 °C. For controls, primary antibodies were omitted. Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit, sheep, and goat IgG, and Texas Red or Rhodamine Red-X-conjugated donkey anti-goat, sheep, and rabbit IgG (Jackson Immunoresearch, West Grove, PA), all at a dilution of 1:200. The secondary antibodies were applied for an hour at room temperature. The slides were then washed with 3 changes of PBS and 1 change of 4 mM sodium carbonate (pH 10.0), cover-slipped, and examined with a conventional fluorescence microscope (Eclipse E800M: Nikon, Melville, NY). The images were captured with a digital camera (SpotCam: Diagnostic Instruments, Sterling Heights, MI) and analyzed on a desktop PC computer with PhotoShop (Adobe Systems, Mountain View, CA). At least 21 sections were examined per gland in order to examine all lobes within the gland.

Sucrose-Potassium Phosphate-Glyoxylic Acid (SPG) Method

The SPG technique, as developed by De la Torre,[19] is a very sensitive and powerful method for visualizing adrenergic nerves.[7,10] Lacrimal glands were quickly removed from the animals and frozen in OCT medium on chucks in the cryostat at –20 °C. Sections (14 μm thick) were cut and picked up on room temperature slides and immediately dipped 3 times for 1 second in the glyoxylic acid solution. The sections were dried with a blow dryer set at cool. When dry, the sections were covered with 2 drop of mineral oil (USP grade) and placed on an aluminum tray in a 95 °C oven for 2 minutes.

The slides were drained of excess oil, covered with 2 drops of fresh oil, and coverslipped. The sections were examined with a conventional fluorescence microscope.

Protein Secretion

Lacrimal glands were weighed before being cut into fragments of 1-2 mm with a scalpel blade. The fragments were washed in 5 ml of saline solution (116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.81 mM $MgCl_2$, 1.01 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 5.6 mM dextrose, pH 7.4) that was maintained at 37 °C and was vigorously bubbled with 95% O_2 and 5% CO_2 in a beaker for 10 minutes. The solution was changed 3 times and discarded. The gland fragments were then incubated in 1 ml saline for 10 minutes. The saline was then removed and replaced with fresh medium. This was repeated for 2 or 3 times and the saline was collected after each of the 10-minute incubations. The proteins in these samples represent the basal secretion from the glands. In the last exchange, the medium that was added contained one of the drugs at a specific concentration. After another 10-minute incubation, the medium was removed and saved, and the proteins in these solutions represented the stimulated secretion in response to various drugs. One gland was used in each experiment.

Although isoproterenol is subject to inactivation by oxidation in oxygenated medium, our relatively short incubation period (10 minutes), did not cause a significant change in concentration.[12] Also, though isoproterenol becomes brownish pink upon oxidition, the color did not become visually perceptable until the concentration exceeded 10^{-4} M. However, to ensure that the discoloration did not decrease accuracy, control

samples with corresponding isoproterenol concentrations were included in each experiment and corrections were made at various concentrations.

Protein Assay

The samples were analyzed for their total protein using a Coomassie protein assay kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard protein, and standards were run with each assay. Protein concentrations were determined from the standard curves measured with each assay. The assays were performed on an EL 808 microplate reader (Bio-Tek Instruments, Winooski, VT), read at 595 nm. Both samples and standards were read in duplicate, using 96-well Costar flat bottom microplates (Corning Inc., Corning, NY). Total protein concentration was determined using the software provided by the manufacturer (KC4; Bio-Tek). Proteins secreted in response to various agonists (stimulated secretion) were the difference between total and basal secretions. The readings were then converted to μg/ml per gram tissue per minute (μg/ml/g/min).

Quantification of VAChT IR

Under low-power magnification, 10 randomly selected rectangular areas (30 μm × 40 μm) in both low- and high-innervation density areas were chosen. Using a 40× objective, the punctate VAChT labeling was then counted, and the numbers in each area were averaged. The averages represent the numbers of VAChT IR in either low- or high-innervation density areas.

Statistical Analysis

Data were expressed as mean ± SEM, wherever appropriate. Student paired or unpaired *t*-tests were performed using SigmaPlot 5.0 software (SPSS Inc., Chicago, IL). $P<0.05$ was considered to be significant.

RESULTS

Sympathetic Innervation in the Gland

Examination of the lacrimal gland stained with antisera to TH (host in either rabbit or sheep) showed that some lobes of the gland had relatively sparse TH immunoreactivity (TH IR; low-ID areas) while other lobes had extensive TH IR (high-ID areas). In all lobes, TH IR was found associated with the blood vessels (Fig. 1A). In the lobes with low-ID, in addition to the TH IR associated with the blood vessels, a few positive fibers were seen among the acini. These fibers were varicose and were close to some acini (Fig. 1B). However, the majority of acini did not have TH IR fibers associated with them. In some cases, there were regions in the low-ID areas that did have a higher density of TH IR, but they were not common (Fig. 1C). In the lobes with high-ID TH IR, there was an extensive network of positively staining fibers. The immunoreactivity was so intense that the fibers appeared smooth with few obvious varicosities (Fig. 1D). In these lobes, virtually every acinar cell had a TH IR fiber close to it. Using serial sections from the whole glands, we found that the high-ID areas were usually situated in the rostral-ventral side of the gland, often beneath low-ID areas of the gland. The size of high-ID areas varied from 10% to 30% of the whole gland.

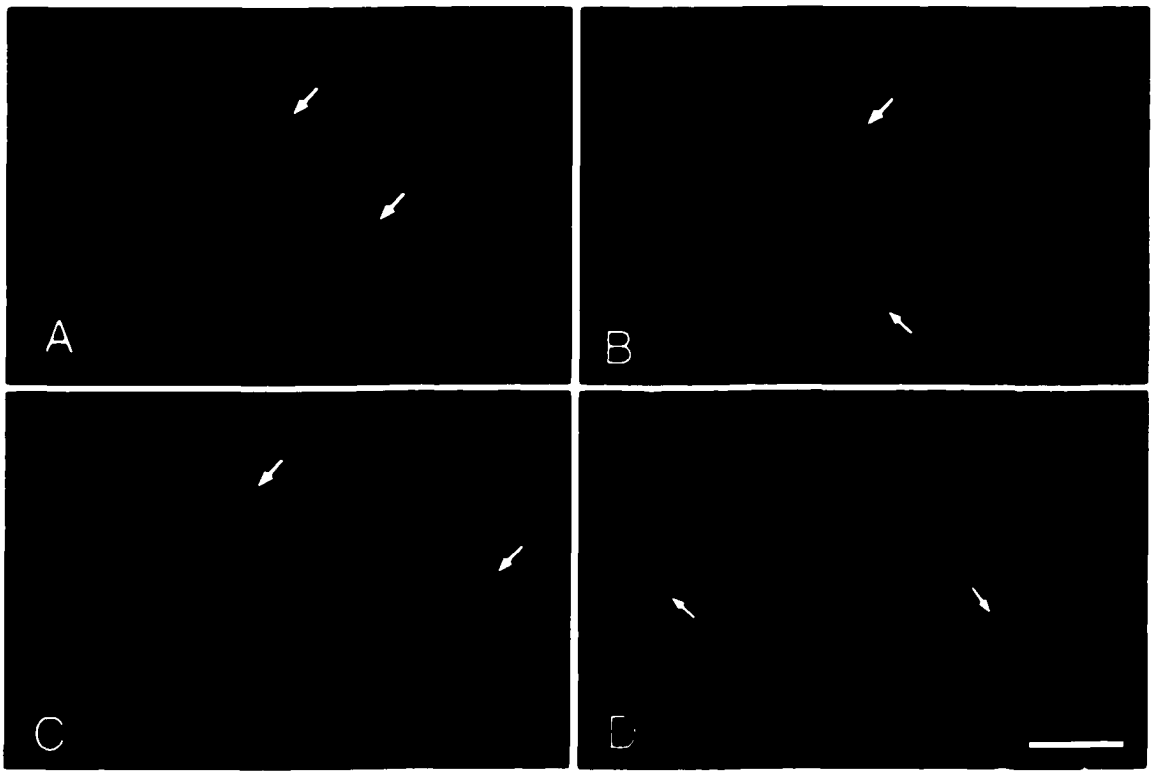

FIGURE 1. Sympathetic innervation in the mouse lacrimal gland. (**A**) TH immunoreactive nerve fibers were observed to be in association with blood vessels (arrows). (**B**) Nerve fibers that were TH immunoreactive were observed to have large number of varicosities and were in close contact with acini in the low-ID areas. (**C**) In some regions within the low-ID areas, TH immunoreactive fibers were observed to be in close association with acini and in relatively higher density than other regions. These regions were mostly found in the periphery of the gland. (**D**) TH IR visualized with FITC in the high-ID areas. Thick nerve bundles and fibers that exhibit TH immunoreactivity to TH appeared smooth and formed dense plexus around acini. Scale bar, 40 μm.

The SPG method revealed a pattern of fibers similar to that observed with the TH antibodies. In all parts of the gland, fluorescent fibers were associated with the blood vessels that are found within the lobes (Fig. 2A). In some lobes, there were occasional varicose fibers among the acini (Fig. 2B), while in other lobes, there was a very dense meshwork of fibers among the acini (Fig. 2C).

Parasympathetic Innervation in the Gland

VAChT IR was found in all lobes of the gland. Varicose fibers were found among acini, presumably representing synaptic vesicles containing acetylcholine (ACh). These VAChT-positive varicosities were close to the basolateral membrane of acini and formed a loose network around them (Fig. 3A). Some larger non-varicose nerve fibers were also observed to be VAChT immunoreactive.

In the lobes of the gland that TH IR staining showed to be high-ID areas, it appeared that more VAChT-positive fibers were also observed in these areas (Fig. 3A). Because VAChT IR was always punctate with obvious varicosities, we were able to quantify VAChT IR in the low-ID and high-ID areas. We estimated that the number of VAChT IR in the high-ID areas was about 3.7 times of the low-ID areas (Fig. 4).

Total Innervation in the Gland

In the low-ID areas, the synaptophysin IR (SIR) appeared to be evenly distributed among most of the acini, although some acini exhibited less SIR. The SIR was characteristically punctate and outlined the acini. Some SIR nerve fibers could also be observed. However, in the high-ID areas, SIR was observed to be of higher density than

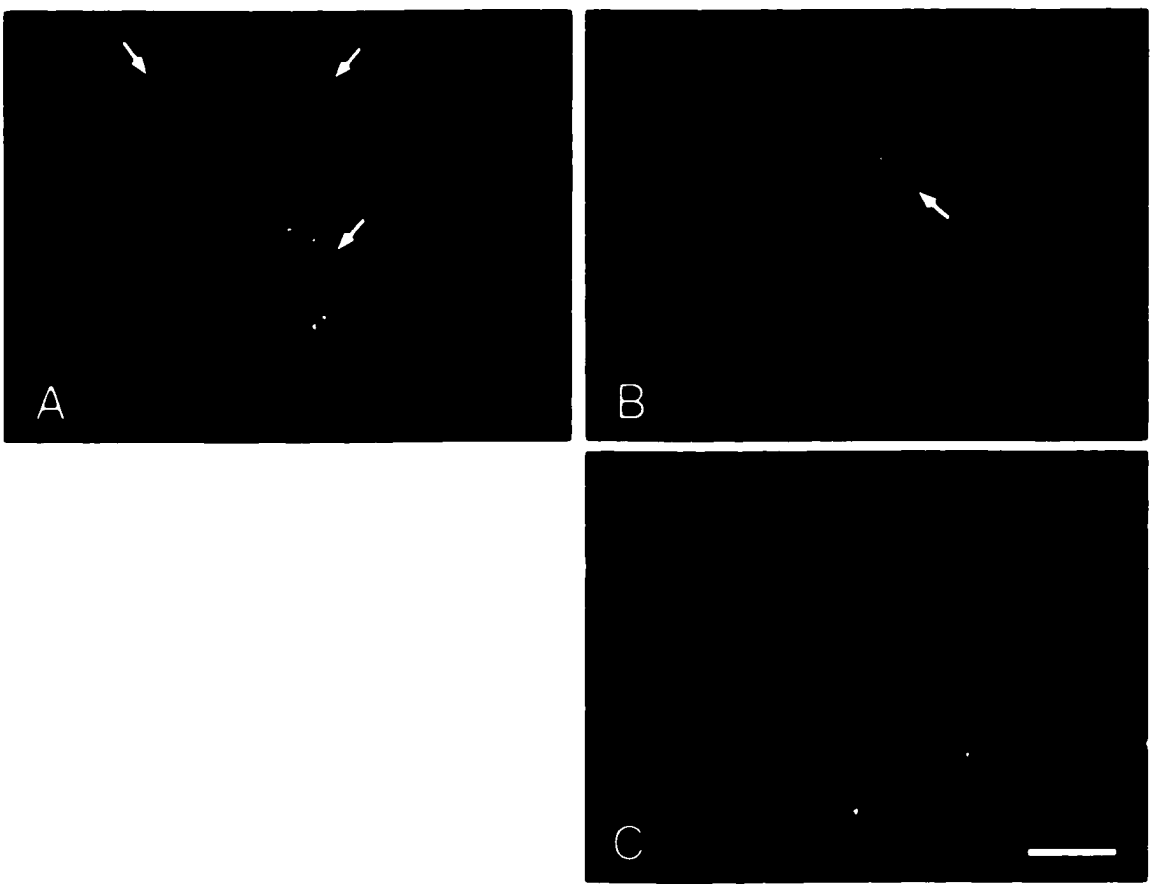

FIGURE 2. Adrenergic innervation in the lacrimal gland as revealed by the SPG method. **(A)** Adrenergic fibers were in close contact with blood vessels. **(B)** In the low-ID areas, sparse nerve fibers were observed in association with acini, along with those around blood vessels (not shown in this figure). **(C)** A dense meshwork of adrenergic fibers was apparent among acini in the high-ID areas. Scale bar, 30 μm.

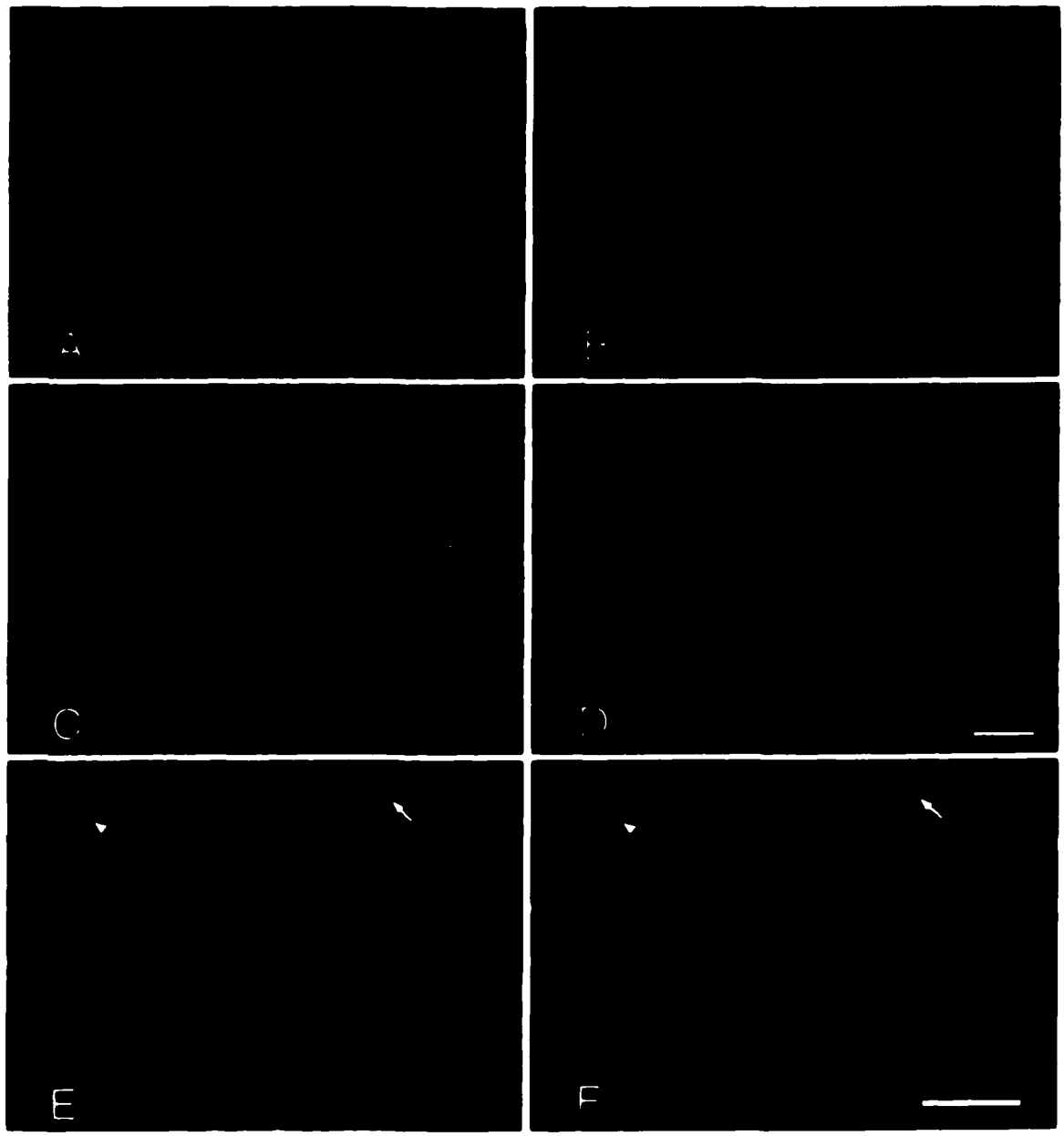

FIGURE 3. Double labeling of the innervation in the lacrimal gland. (**A**) VAChT IR was observed in both low- and high-ID areas. (**B**) Synaptophysin IR (SIR) colocalized with all VAChT IR in both low- and high-ID areas. (**C**) Dense TH immunoreactive nerve fibers were observed in the high-ID areas, mostly in thick bundles of fibers with a smooth appearance. No TH IR could be visualized in low-ID areas in this section. (**D**) Dense SIR (with FITC) observed in the high-ID areas colocalized with TH IR, whereas SIR in the low-ID areas did not. Note that even in the low-ID areas, there were regional differences in the SIR density. Scale bar in A, B, C, and D, 100 μm. (**E**) In high-ID areas, some VAChT IR colocalized or be in close contact with TH IR (arrows). (**F**), and one nerve bundle appeared to include both VAChT IR and TH IR fibers, suggesting that these nerves contain both parasympathetic and sympathetic fibers. Scale bar in E and F, 40 μm.

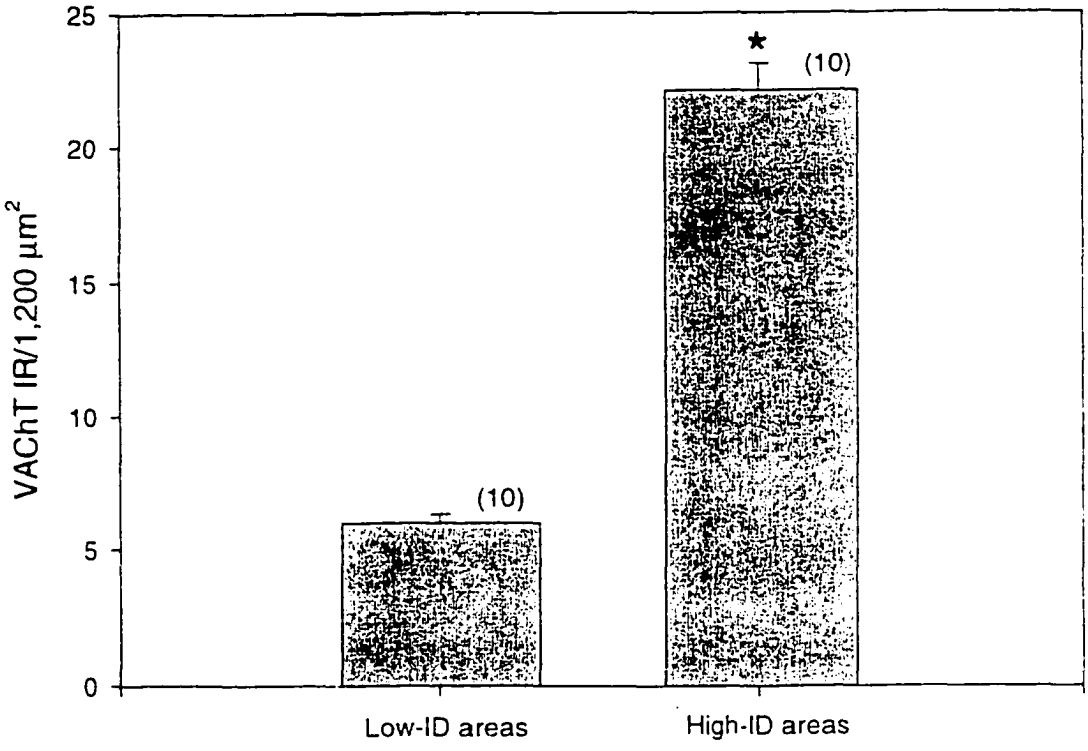

FIGURE 4. The regional differences of VAChT IR in the mouse lacrimal gland. Values are means ± SEM. Numbers of sampling areas are in parentheses. Each sampling area was 30 µm × 40 µm = 1,200 µm^2. *Indicates significant difference from low-ID area.

that in lower-ID areas. Most SIR nerve fibers were thick and had smooth surfaces, displaying morphological properties similar to those observed with the TH antibody, and the punctate SIR appeared to merge, forming a dense plexus of nerve fibers (Figs. 3B, 3D).

Double Labeling

Double labeling showed that in the high-ID areas, the majority of SIR fibers also exhibited TH IR (Figs. 3C, 3D). Some VAChT IR nerves also showed TH IR (Figs. 3E, 3F), suggesting that nerves in this area are mixed, containing both parasympathetic and sympathetic nerve fibers. However, it appeared that the sympathetic innervation predominated in the high-ID areas.

In the low-ID areas, most of the SIR colocalized with VAChT IR (Figs. 3A, 3B), suggesting that most of the innervation of these lobes was of parasympathetic origin, with the exception of a few nerve fibers that were TH IR in the interstitial areas and blood vessels.

Protein Secretory Responses to Autonomic Agonists at 10^{-5} M

The immunohistochemical studies raised the possibility that the sympathetic system may play a major role in the regulation of lacrimal gland function in the mouse. In order to test this possibility, we exposed lacrimal gland fragments to adrenergic agonists and measured the amount of protein secreted in response to each drug. Norepinephrine (α- and β-adrenergic receptors agonist), phenylephrine (α_1-adregergic agonist), and isoproterenol (β-adrenergic agonist) all induced protein secretion from the gland

fragments (Fig. 5). For comparison, we also used carbachol, which is a muscarinic cholinergic agonist.

Norepinephrine, carbachol, and phenylephrine induced the largest secretion, whereas isoproterenol induced the smallest secretion (Table 1). Norepinephrine and phenylephrine elicited almost the same magnitude of protein secretion as that elicited by carbachol ($P>0.05$, no significant difference between any two of them). Isoproterenol appeared to be a relatively weaker stimulant, as the increase in protein secretion was significantly different from the increases induced by the other three agonists.

TABLE 1. Protein Secretion in Response to Autonomic Agonists at 10^{-5} M

Agonist	Stimulated Secretion*	*P* value†
Norepinephrine	336.13	< 0.01
Phenylephrine	301.28	< 0.01
Carbachol	319.10	< 0.01
Isoproterenol	80.78	

*Stimulated secretion (μg/ml/g/min) = total secretion in response to agonist – basal secretion. †Differences between isoproterenol-induced secretion and those induced by other three agonists.

DISCUSSION

The sympathetic innervation varies greatly among species, and there are some discrepancies between reported observations. In rats[5,10] and mice,[5,9,16] only sparse adrenergic nerve fibers were observed, and most of them were associated with blood vessels, with only a few found among acini. In guinea pigs, some researchers reported rich adrenergic innervation in the lacrimal gland,[10] while others described a virtual absence in the gland except along the blood vessels.[5] Our observations of TH IR in the

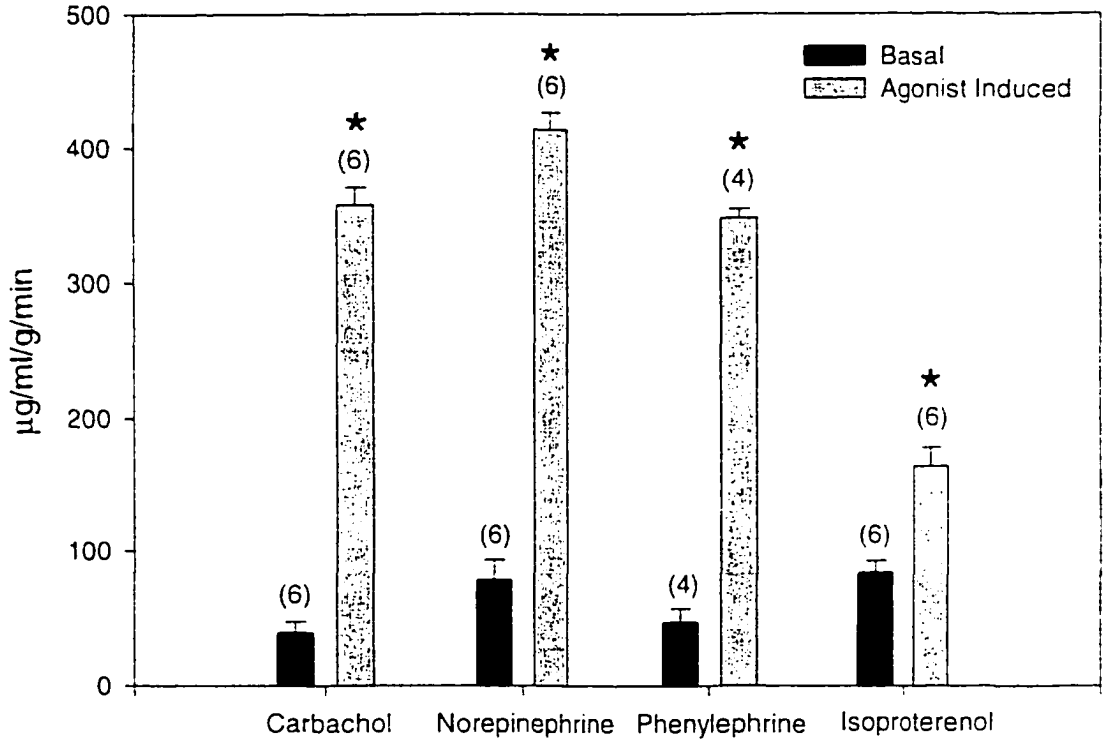

FIGURE 5. Protein secretory responses of the mouse lacrimal gland to agonists at 10^{-5} M. Ordinate is total protein secretion rate (µg/ml per gram tissue per minute). Values are means ± SEM. Numbers in parentheses are replicates of each experiment. *Indicates significant difference from their corresponding basal secretion rate of each agonist. There are no significant differences between any two responses evoked by carbachol, norepinephrine, and phenylephrine. The secretions induced by isoproterenol, however, is significantly lower than those induced by carbachol, norepinephrine, and phenylephrine.

low-ID areas appeared to be in agreement with previous reports in the mouse,[5,9,16] whereas the observation of TH IR in the high-ID areas is similar to the sympathetic innervation observed in guinea pig extraorbital lacrimal gland.[10] However, the division of the mouse lacrimal gland into two areas, based on the differences of innervation pattern and density, is a novel finding. By using serial sections from the whole gland, we estimated that the high-ID areas represent about 10-30% of the gland and is situated on the rostral side of the gland, mostly near the ventral side. The low-ID areas clearly showed blood vessels with associated staining which suggests that these areas were not simply inaccessible to the antisera. Further, using a totally different technique, the SPG method which has been shown to be very sensitive in visualizing adrenergic nerves,[7,10] we found similar differences in the adrenergic innervation density between different lobes.

Increasing evidence in some species[12,13] indicates that the sympathetic nervous system plays a role in influencing tear secretion not only by modulating blood flow to the gland and its distribution within it, but also by direct effects on the secretory acini. In the present study, the observation of TH-immunoreactive nerve fibers with numerous varicosities in close association with acini in some lobes of the mouse gland support this notion. Axonal varicosities have been shown to represent synaptic junctions. This observation contrasts with previous data from mouse lacrimal gland, which showed extensive adrenergic innervation of blood vessels, but only sparse nerve fibers associated with the secretory acini.[5,9,16]

The protein secretion experiments reported here, using norepinephrine as the agonist for both α- and β-adrenergic receptors, and phenylephrine and isoproterenol for

α_1 and β adrenergic receptors,[12,20] respectively, suggested the presence of both α_1 and β adrenergic receptors in the mouse lacrimal gland and that they are associated with the protein secretion process. The robust responses elicited by norepinephrine and phenylephrine suggest that the sympathetic innervation of the mouse lacrimal gland has more functional significance than previously thought. While there are TH IR fibers associated with the blood vessels within the lacrimal gland, the protein secretion data described above were obtained from *in vitro* lacrimal gland fragments and could not be caused by the modulation of blood flow in the gland. The significant response of the gland fragments to adrenergic agonists suggests there is extensive direct adrenergic control of acinar cells.

In the mouse lacrimal gland, there was α_1 adrenergic-mediated stimulated protein secretion. Phenylephrine has been shown to increase intracellular Ca^{2+} concentrations[21] and induce significant peroxidase secretion (an index of protein secretion) in the mouse.[22] Intracellular recordings of mouse acinar cells indicate that epinephrine elicited hyperpolarization and a marked reduction of membrance resistance.[23,24] In the rat lacrimal gland, phenylephrine stimulates protein secretion from acini in a time- and concentration-dependent manner, with the maximum reached at 10^{-4} M.[25] α adrenergic agonists, such as phenylephrine and norepinephrine, can induce K^+ release and secretion of both peroxidase and newly synthesized protein from rat acinar cells.[13,25-27]

The selective α_1 adrenergic agonist phenylephrine appears to have intrinsic activity similar to that of norepinephrine, a mixed α and β receptor agonist, in eliciting protein secretion. In rats, 85% of the adrenergic regulation of protein secretion was achieved through α_1 adrenergic receptor activation, and the remaining 15% was assumed

to be due to the activation of β receptor.[20] Our data are in agreement with those from other studies of rats. At 10^{-5} M, protein secretion induced by phenylephrine is about 89% of that induced by norepinephrine, suggesting that the adrenergic stimulation in the mouse lacrimal gland is also mainly achieved through α_1 receptor activation.

Protein secretion data suggest that a β adrenergic pathway is present in the mouse lacrimal gland as well. Although the isoproterenol-induced protein secretion was much smaller than that induced by other agonists, it doubled the secretion rate. These results appear to contradict a report that the sympathetic influence in mouse lacrimal gland is mediated only by α_1 adrenoceptors.[28] Intracellular recording of mouse lacrimal gland acinar cells, for example, revealed that isoproterenol had no detectable effects on membrane potential and resistance.[23,24] However, in the lacrimal gland of the rat, isoproterenol stimulates both fluid and protein (peroxidase) secretion, though the response is small and no K^+ release could be observed.[13,29,30] *In vivo* experiments on cannulated rabbit lacrimal gland also demonstrated that there are norepinephrine-responsive β adrenergic receptors present, and that isoproterenol was more effective than norepinephrine and epinephrine in inducing lacrimal flow.[31] Reports from *in vitro* studies using rabbit lacrimal gland fragments also show that isoproterenol can induce protein secretion.[12]

Although there were no significant differences between protein secretion rates induced by norepinephrine, phenylephrine, and carbachol, the significant increase in protein secretion induced by norepinephrine and phenylephrine appears to be in contrast to the prevailing view that the parasympathetic system is responsible for most of the protein secretion in the mouse lacrimal gland. Previous studies reported that there was

only sparse sympathetic innervation of the mouse lacrimal gland[5,9,16] and that there is much more parasympathetic innervation present in the gland. These reports suggested that the sympathetic innervation observed might not be sufficient to induce robust protein secretion in response to adrenergic agonists.

The sympathetic nerves within the lacrimal gland primarily originate from the superior cervical ganglion (SCG), which is the uppermost ganglion of the sympathetic trunk. In the lacrimal gland of rats,[32] retrograde-tracing experiments showed both ipsi- and contralateral sympathetic contributions from SCG neurons, though only a few labeled somata were observed in the contralateral SCG. Although no comparable data concerning the sympathetic source in mouse lacrimal gland is available, it seems likely that the mouse lacrimal gland may be similar to that of the rat, with both ipsi- and contralateral SCG contributions. We speculate that there may be some relationship between the origin of the sympathetic input to the lacrimal gland and the innervation density; i.e., perhaps the sympathetic innervation in the high-ID areas comes from the ipsi-lateral SCG, while that in the low-ID areas originates contralaterally, or vice versa. Retrograde labeling studies, with dye injections into either the high- or low-ID areas of the lacrimal gland, and subsequent evaluation of the labeling in the ipsi- and contralateral SCG could test this idea.

In addition to TH IR, regional differences of innervation were also observed in both synaptophysin and VAChT immunoreactivity: much more synaptophysin and VAChT immunoreactivity was observed in the high-ID areas. Synaptophysin has been well documented as a marker of total innervation,[8] and VAChT has been extensively documented as a marker for parasympathetic nerves.[17,18] Our data suggest that the mouse

lacrimal gland is divided into two areas, one with much higher innervation density (both parasympathetic and sympathetic), and the other with lower density. By counting the number of VAChT IR puncta, it was estimated that the parasympathetic innervation in the high-ID areas is about 3.7 times that of the low-ID area. This is a novel observation that contrasts with data from studies of the autonomic supply to lacrimal glands in mice,[5,9] rats,[11] humans and monkeys,[6] which showed relatively even innervation density.

The regional differences of innervation suggest that the mouse lacrimal gland is a mixed gland. In the high-ID areas, in which dense nerve fibers were observed around blood vessels and acini, the density and distribution patterns of synaptophysin and TH immunoreactivity were similar to the sympathetic innervation in guinea pig extra- and intra-orbital glands.[10] In the low-ID areas, however, the TH IR density was similar to the sympathetic innervation observed in lacrimal glands of rats[10] and mice.[5,9] This is reminiscent of the innervation pattern in avian Harderian gland, the main lacrimal gland in birds, which has two anatomically distinct areas, the cortex and the medulla. The cortex consists of secretory tubules made of columnar epithelium, whereas the medulla contains only a few. Both cholinergic and adrenergic nerves are observed in the Harderian gland, but only sparse fibers are observed around the cortical secretory acini in pigeons[33] and chickens,[34,35] in contrast to the dense innervation observed in the medulla.

Anatomically, the mouse exorbital lacrimal gland consists of several lobes, and each lobe has a duct that runs to the surface of the eye. There are 6 to 7 ducts that come together to form the main duct. Therefore, it is conceivable that the lobes have different innervation patterns and functions. A lobe itself consists of many lobules, which are further comprised of acini, the functional units of tear secretion. Since tear flow is highly

regulated, division of the mouse lacrimal gland into two distinct areas according to the differences of innervation density and distribution pattern suggests that the two areas may have different functions.

In addition to classical neurotransmitters such as ACh and norepinephrine, various neuropeptides[14,15,36-38] and other unconventional transmitters such as nitric oxide[16] have been found in the mammalian lacrimal gland, including that of the mouse. The available data suggest that both classical neurotransmitters and at least two neuropeptides are present in the lacrimal glands of most species.[35,36]

The complexity of the innervation pattern of the lacrimal gland, in conjunction with the multiple neurotransmitters and modulators present, may influence the protein, ion, and water composition of tears.[12,35] Based on the results obtained from rabbit, Bromberg et al. suggested that both parasympathetic and sympathetic systems work together to effect secretion of tears of the appropriate composition.[12] For example, the parasympathetic system may regulate the flow rate and electrolyte content, and the sympathetic system may regulate protein secretion.[12] Differential secretion could be accomplished by stimulating different autonomic nervous pathways in acinar and ductal cells, by activating different populations of secretory cells, or by the activation of different intracellular signal systems. There is precedence for this idea.

Based upon data from muscarinic ACh receptor immunohistochemical observations in the rat lacrimal gland, it was reported that even though the immunoreactivity was associated with each acinus, the labeling appeared to be unevenly distributed both within an acinus and between them.[35] The two separate areas within the mouse lacrimal gland, with distinctive innervation densities and distribution patterns, and

the regional differences of innervation even in the low-ID areas, suggest that the secretory cells in these two areas may be differentially stimulated. Tears of varying composition, resulting from differential sympathetic and parasympathetic stimulation, could thus be produced depending on the needs of a given situation.

It is unlikely that we observed a pathological condition because the animals used, aged between 2 to 12 months, were healthy at the time of experiment. Tissues obtained from many animals yielded similar results, and it was very unlikely that all these animals had the same pathological condition.

It must be pointed out that because our observations were made in the course of a study of Sjögren's syndrome, we used only female mice, so the data reported here may not be applicable to male mice. Another point is that the preparation used for protein secretion was from fragments of the lacrimal gland, which may have contained nerve fibers, ductal cells, myoepithelial cells, mast cells, and plasma cells. However, as acinar cells represent at least 80% of the rat lacrimal gland mass,[13,39,40] we believe the data presented here are representative of the acinar cells.

In summary, these data indicated that there is extensive sympathetic innervation in the mouse lacrimal gland and that it appears to be directly associated with the protein secretory process. Immunohistochemistry and SPG observations suggest the mouse lacrimal gland can be divided into two areas based on the innervation density and distribution pattern. One area, comprising about 10% to 30% of the gland, has higher innervation density, both parasympathetic and sympathetic. The rest of the gland is less densely innervated. These two areas may play different roles in secreting tears that are of different compositions in various situations. These data support the notion that

differential secretion is accomplished by activating different populations of secretory cells, which are differentially innervated.

References

1. Botelho SY, Hisada M, Fuenmayor N. Functional innervation of the lacrimal gland in the cat. Origin of secretomotor fibers in the lacrimal nerve. *Arch Ophthalmol.* 1966;76:581-588.

2. Ruskell GL. Changes in nerve terminals and acini of the lacrimal gland and changes in secretion induced by autonomic denervation. *Z Zellforsch Mikrosk Anat.* 1969;94:261-281.

3. Ruskell GL. The distribution of autonomic post-ganglionic nerve fibers to the lacrimal gland in monkeys. *J Anat.* 1971;109:229-242.

4. Ehinger B. Distribution of adrenergic nerves in the eye and some related structures in the cat. *Acta Physiol Scand.* 1966;66:123-128.

5. Ehinger B. Ocular and orbital vegetative nerves. *Acta Physiol Scand Suppl.* 1966;268:1-35.

6. Ehinger B. Adrenergic nerves to the eye and to related structures in man and in the cynomolgus monkey (Macaca irus). *Invest Ophthalmol.* 1966;5:42-52.

7. Powell CC, Martin CL. Distribution of cholinergic and adrenergic nerve fibers in the lacrimal glands of dogs. *Am J Vet Res.* 1989;50:2084-2088.

8. Walcott B, Claros N, Patel A, Brink PR. Age-related decrease in innervation density of the lacrimal gland in mouse models of Sjogren's syndrome. *Adv Exp Med Biol.* 1998;438:917-923.

9. Zoukhri D, Hodges RR, Dartt DA. Lacrimal gland innervation is not altered with the onset and progression of disease in a murine model of Sjogren's syndrome. *Clin Immunol Immunopathol.* 1998;89:126-133.

10. Nikkinen A, Uusitalo H, Lehtosalo JI, Palkama A. Distribution of adrenergic nerves in the lacrimal glands of guinea-pig and rat. *Exp Eye Res.* 1985;40:751-756.

11. Walcott B, Cameron R, Grine E, Roemer E, Pastor M, Brink PR. Anti-muscarinic acetylcholine receptor-like immunoreactivity in lacrimal glands. *Adv Exp Med Biol.* 1994;350:61-65.

12. Bromberg BB. Autonomic control of lacrimal protein secretion. *Invest Ophthalmol Vis Sci.* 1981;20:110-116.

13. Parod RJ, Putney JW, Jr. An alpha-adrenergic receptor mechanism controlling potassium permeability in the rat lacrimal gland acinar cell. *J Physiol.* 1978;281:359-369.

14. Seifert P, Stuppi S, Spitznas M, Weihe E. Differential distribution of neuronal markers and neuropeptides in the human lacrimal gland. *Graefes Arch Clin Exp Ophthalmol.* 1996;234:232-240.

15. Matsumoto Y, Tanabe T, Ueda S. Kawata M. Immunohistochemical and enzymehistochemical studies of peptidergic, aminergic and cholinergic innervation of the lacrimal gland of the monkey (Macaca fuscata). *J Auton Nerv Syst.* 1992;37:207-214.

16. Ding C, Walcott B, Keyser KT. Neuronal nitric oxide synthase and the autonomic innervation of the mouse lacrimal gland. *Invest Ophthalmol Vis Sci.* 2001;42:2789-2794.

17. Arvidsson U, Riedl M, Elde R, Meister. Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. *J Comp Neurol.* 1997;378:454-467.

18. Gilmor ML, Nash NR, Roghani A, et al. Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. *J Neurosci.* 1996;16:2179-2190.

19. De la Torre JC. An improved approach to histofluorescence using the SPG method for tissue monoamines. *J Neurosci Methods.* 1980;3:1-5.

20. Mauduit P, Herman G, Rossignol B. Protein secretion in lacrimal gland: alpha 1-beta-adrenergic synergism. *Am J Physiol.* 1986;250:C704-C712.

21. Zoukhri D, Hodges RR, Rawe IM, Dartt DA. Ca^{2+} signaling by cholinergic and alpha1-adrenergic agonists is up-regulated in lacrimal and submandibular glands in a murine model of Sjogren's syndrome. *Clin Immunol Immunopathol.* 1998;89:134-140.

22. Zoukhri D, Kublin CL. Impaired neurotransmitter release from lacrimal and salivary gland nerves of a murine model of Sjogren's syndrome. *Invest Ophthalmol Vis Sci.* 2001;42:925-932.

23. Iwatsuki N, Petersen OH. Membrane potential, resistance, and intercellular communication in the lacrimal gland: effects of acetylcholine and adrenaline. *J Physiol.* 1978;275:507-520.

24. Iwatsuki N, Petersen OH. Intracellular Ca^{2+} injection causes membrane hyperpolarization and conductance increase in lacrimal acinar cells. *Pflugers Arch.* 1978;377:185-187.

25. Hodges RR, Dicker DM, Rose PE, Dartt DA. Alpha 1-adrenergic and cholinergic agonists use separate signal transduction pathways in lacrimal gland. *Am J Physiol.* 1992;262:G1087-G1096.

26. Thorig L, van Haeringen NJ, Timmermans PB, van Zwieten PA. Alpha-adrenoceptor control of peroxidase secretion from rat lacrimal gland cells in vitro. *Exp Eye Res.* 1982;35:29-41.

27. Putney JW Jr, VandeWalle CM, Leslie BA. Stimulus-secretion coupling in the rat lacrimal gland. *Am J Physiol.* 1978;235:C188-C198.

28. Qi JH, Li BH. Effects of alpha adrenoceptor agonists and antagonists on palpebral fissure and lacrimation in mice. *Zhongguo Yao Li Xue Bao.* 1992;13:153-156.

29. Mauduit P, Herman G, Rossignol B. Protein secretion induced by isoproterenol or pentoxifylline in lacrimal gland: Ca^{2-} effects. *Am J Physiol.* 1984;246:C37-C44.

30. Friedman ZY, Lowe M, Selinger Z. Beta-Adrenergic receptors stimulated peroxidase secretion from rat lacrimal gland. *Biochim Biophys Acta.* 1981;675:40-45.

31. Botelho SY, Goldstein AM, Martinez EV. Norepinephrine-responsive beta-adrenergic receptors in rabbit lacrimal gland. *Am J Physiol.* 1973;224:1119-1122.

32. Ten Tusscher MP, Klooster J, Baljet B, Van der Werf F, Vrensen GF. Pre- and post-ganglionic nerve fibres of the pterygopalatine ganglion and their allocation to the eyeball of rats. *Brain Res.* 1990;517:315-323.

33. Walcott B, McLean JR. Catecholamine-containing neurons and lymphoid cells in a lacrimal gland of the pigeon. *Brain Res.* 1985;328:129-137.

34. Walcott B, Sibony PA, Keyser KT. Neuropeptides and the innervation of the avian lacrimal gland. *Invest Ophthalmol Vis Sci.* 1989;30:1666-1674.

35. Walcott B, Cameron RH, Brink PR. The anatomy and innervation of lacrimal glands. *Adv Exp Med Biol.* 1994;350:11-18.

36. Nikkinen A, Lehtosalo JI, Uusitalo H, Palkama A, Panula P. The lacrimal glands of the rat and the guinea pig are innervated by nerve fibers containing immunoreactivities for substance P and vasoactive intestinal polypeptide. *Histochemistry.* 1984;81:23-27.

37. Williams RM, Singh J, Sharkey KA. Innervation and mast cells of the rat exorbital lacrimal gland: the effects of age. *J Auton Nerv Syst.* 1994;47:95-108.

38. Sibony PA, Walcott B, McKeon C, Jakobiec FA. Vasoactive intestinal polypeptide and the innervation of the human lacrimal gland. *Arch Ophthalmol.* 1988;106:1085-1088.

39. Dartt DA. Signal transduction and control of lacrimal gland protein secretion: a review. *Curr Eye Res.* 1989;8:619-636.

40. Herzog V, Sies H, Miller F. Exocytosis in secretory cells of rat lacrimal gland. Peroxidase release from lobules and isolated cells upon cholinergic stimulation. *J Cell Biol.* 1976;70:692-706.

THE α_1 AND β_1 ADRENERGIC MODULATION OF LACRIMAL GLAND FUNCTION IN THE MOUSE

by

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ABSTRACT

PURPOSE. To determine the express patterns of α_1 and β_1 adrenergic receptors in the mouse lacrimal gland. α and β adrenergic receptor agonist and antagonist were used to elucidate the receptors' relevance to protein secretion.

METHODS. Mouse lacrimal glands were processed for single- and double-labeled indirect immunofluorescence studies and examined with confocal scanning microscopy. Protein secretion and SDS-PAGE experiments were also employed.

RESULTS. Extensive α_1 and β_1 adrenergic receptor immunoreactivity was observed in the mouse lacrimal gland. The protein secretion in response to phenylephrine and isoproterenol showed that direct stimulation of either α_1 or β_1 adrenergic receptor could induce significant protein secretion from the lacrimal gland. The specificity of this stimulation was further indicated by the effects of adrenergic receptor antagonists. Preliminary SDS-PAGE results showed that different proteins were secreted in response to α_1 and β agonists. No synergism was observed between α_1 and β receptor-mediated protein secretions.

DISCUSSIONS. Our results support the notion that there is extensive adrenergic control in the mouse gland. We propose that the adrenergic receptors may be a better choice of markers to reflect the extent of adrenergic control, because circulating norepinephrine in the blood stream should be taken into consideration. Both confocal microscopy observations and protein secretion data suggest the presence of α_1 and β_1 adrenergic receptor-mediated pathways in the mouse lacrimal gland. Preliminary data appeared to support the existence of differential secretion in the mouse lacrimal gland.

INTRODUCTION

The adrenergic system in the mammalian lacrimal gland has been assumed to play an indirect and minor role in lacrimal function, mostly by regulating the blood flow and distribution to the gland.[1,2] However, increasing evidence indicates that the adrenergic system may also play a direct and significant role in regulating tear secretion,[3,4] suggesting the lacrimal gland may be under the control of both cholinergic and adrenergic innervation.

Electrophysiological recordings from mouse lacrimal acinar cells showed that isoproterenol markedly enhances ATP-induced currents, which can be blocked by propranolol.[5] Iontophoretic application of epinephrine to mouse lacrimal acinar cells increases potassium permeability, which is similar to the change induced by acetylcholine.[6] Similar findings were obtained from rats.[7] In rabbits, adrenergic stimulation has been shown to enhance lacrimal flux.[1,2,8] These data suggest that the adrenergic stimulation may have a direct and significant influence on lacrimal acinar cells.

Most previous investigations have used markers such as tyrosine hydroxylase or dopamine β-hydroxylase to visualize the adrenergic innervation in the lacrimal gland.[9,10,11] However, catecholamines released by the adrenergic fibers in the lacrimal gland proper are not the only source. Catecholamines are also released by other tissues or organs into the blood stream and serve as systemic neurotransmitters and may diffuse from the blood stream into the lacrimal gland milieu and influence secretory processes.

For those acini that are in close association with adrenergic nerves, catecholamines released from nerve terminals may influence secretory cells directly.

However, as catecholamines are inactivated quickly upon release from the terminals, it seems unlikely that the catecholamines would diffuse across long distances to the acinar cells and still reach the threshold concentration to stimulate secretion. Therefore the assumption that the adrenergic nerves present in the lacrimal gland are the sole source of catecholamines in the gland may be unwarranted. Adrenergic receptors may be better indicators to reflect the extent of adrenergic control. However, we were unable to find any published report of the direct observation of adrenergic receptors in the lacrimal gland of any species. Therefore, in the present study, we used antibodies against α_1 and β_1 adrenergic receptors to determine the distribution of these receptors in the mouse lacrimal gland. We also studied the protein secretory responses induced by adrenergic agonists.

Our data suggest that every acinar cell has a large number of α_1 and β_1 adrenergic receptors. Protein secretion results indicate that the adrenergic system plays a direct and significant role in modulating protein secretion from the mouse lacrimal gland. Our studies also suggest that the proteins secreted in response to phenylephrine and isoproterenol stimulation were not the same, providing evidence of the existence of differential secretion.

MATERIALS AND METHODS

Chemicals

Carbamylcholine chloride (carbachol), isoproterenol bitartrate, norepinephrine bitartrate, $_{L}$-phenylephrine hydrochloride, phentolamine hydrochloride, and $_{DL}$-

propranolol hydrochloride were from Sigma (St. Louis, MO). All reagents were of the highest purity available.

Animals

C57 female mice (~18 g body weight), aged from 2 to 12 months, were purchased from commercial vendors (Taconic Farms, Germantown, NY, or Charles River, Wilmington, MA). All animals were kept in a 12-hour light-dark cycle and maintained in an accredited animal facility with freely available food and water. They were managed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To obtain lacrimal glands, the animals were sacrificed with an overdose of halothane around midday, and lacrimal glands were rapidly removed and trimmed from surrounding membranes and fatty tissues under a dissecting microscope.

Tissue Preparation for Immunohistochemistry

For immunohistochemistry, lacrimal glands were fixed in freshly prepared 4% buffered paraformaldehyde. After 3-4 hours of fixation at 4 °C, the tissue was placed in 0.1 M phosphate buffer containing 30% sucrose at pH 7.4 for at least 12 hours at 4 °C. The glands were then placed in "optimal cutting temperature" (OCT) embedding medium (Sakura Finetek USA, Torrance, CA), serially sectioned at 10 μm on a cryostat (Leica, Deerfield, IL), and collected on slides (SuperFrost Plus; VWR Scientific, West Chester, PA). The sections were dried and stored at −20 °C until used.

Immunohistochemistry

The antibodies used were rabbit anti-α_1 adrenergic receptor polyclonal antibody (Oncogene Research Products, San Diego, CA), at a dilution of 1:100; rabbit anti-β_1 adrenergic receptor polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), at a dilution of 1:1000; and sheep anti-tyrosine hydroxylase (TH) polyclonal antibody (Chemicon International, Temecula, CA), at a dilution of 1:800.

Sections were incubated in primary antibody diluted with 0.1 M sodium phosphate buffer (PBS) for 48 hours at 4 °C. For controls, primary antibodies were omitted. Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG, at a dilution of 1:100; and Rhodamine Red-X-conjugated donkey anti-sheep IgG (Jackson Immunoresearch, West Grove, PA), at a dilution of 1:200. The secondary antibodies were applied for an hour at room temperature. The slides were then washed with three changes of PBS and one change of 4 mM sodium carbonate (pH 10.0), cover-slipped, and examined with a conventional fluorescence microscope (Eclipse E800M; Nikon, Melville, NY). The images were captured with a digital camera (Spot; Diagnostics Instruments, Sterling Heights, MI), and analyzed on a desktop PC computer with image analysis software (PhotoShop; Adobe Systems, Mountain View, CA). Additional images were also obtained with a confocal laser scanning microscope (Leica; TCS SP). At least 20 sections were examined per gland in order to examine all lobes within the gland.

Protein Secretion

Lacrimal glands were weighed before being cut into fragments of 1-2 mm with a scalpel blade. The fragments were washed in 5 ml of saline solution (116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.81 mM $MgCl_2$, 1.01 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 5.6 mM dextrose, pH 7.4) that was maintained at 37 °C and were vigorously bubbled with 95% O_2 and 5% CO_2 in a beaker for 10 minutes. The solution was changed three times and discarded. The gland fragments were then incubated in 1 ml saline for 10 minutes. The saline was then removed and replaced with fresh medium. This was repeated two or three times, and the saline was collected after each of the 10-minute incubations. The proteins in these samples represent the basal secretion from the glands. In the last exchange, the medium that was added contained one of the drugs at a specific concentration. After another 10-minute incubation, the medium was removed and saved, and the proteins in these solutions represented the stimulated secretion in response to various drugs. One gland was used in each experiment.

Although isoproterenol is subject to inactivation by oxidation in oxygenated medium, our relatively short incubation period (10 minutes) did not cause a significant change in concentration.[3] Also, though isoproterenol becomes brownish pink upon oxidition, the color did not become visually perceptible until the concentration exceeded 10^{-4} M. However, to ensure that the discoloration did not decrease accuracy, control samples with corresponding isoproterenol concentrations were included in each experiment, and corrections were made at various concentrations.

Theoretical additivity was the sum of the experimental responses obtained in the presence of each agonist alone with the basal value subtracted. A response was defined as

additive if the theoretical additivity did not differ significantly from the experimental additivity. This was interpreted to mean that the effects of the two agonists are modulated by separate pathways, but that the pathways interacted. A response was not considered additive if the experimental additivity was significantly lower than the theoretical additivity. In this instance, our interpretation was that the two agonists activated the same pathway.[12]

Protein Assay

The samples were analyzed for their total protein using a Coomassie protein assay kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard protein, and standards were run with each assay. Protein concentrations were determined from the standard curves measured with each assay. The assays were performed on an EL 808 microplate reader (Bio-Tek Instruments, Winooski, VT), read at 595 nm. Both samples and standards were read in duplicate, using 96-well Costar flat bottom microplates (Corning Inc., Corning, NY). Total protein concentration was determined using the software provided by the manufacturer (KC4; Bio-Tek). Proteins secreted in response to various agonists (stimulated secretion) were the difference between total and basal secretions. The readings were then converted to μg/ml per gram tissue per minute (μg/ml/g/min).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were subjected to SDS-PAGE in 10% (vol/vol) polyacrylamide gels (0.75 mm) using a commercial system (Mini-Protean II; Bio-Rad, Hercules, CA). Protein

samples, obtained from gland fragments incubated in the presence of individual agonists, were run in separate gel lanes under reduced (2-mercaptoethanol) and non-reduced conditions. Gels were resolved at 200 V for 45 minutes, stained with GelCode Silver Staining Reagent (Pierce, Rockford, IL). Gels were dried using an automated gel dryer (Bio-Rad, Hercules, CA). The protein concentration of the samples were determined with the Pierce Commassie protein assay kit.

Statistical Analysis

Data were expressed as mean ± SEM, wherever appropriate. Student paired or unpaired *t*-tests were performed using SigmaPlot 5.0 software (SPSS Inc., Chicago, IL). $P<0.05$ was considered to be significant.

RESULTS

Immunohistochemistry Observations

In the lacrimal gland, extensive α_1 and β_1 adrenergic receptor immunoreactivity (IR) was observed. Some α_1 adrenergic receptor IR was observed to be on the surface of acinar cells, while much of the immunoreactivity appeared to be inside the acinar cells (Figs. 1B, 1C). In addition to the α_1 adrenergic receptors that were associated with acinar cells, much more α_1 adrenergic receptor IR was observed in the interstitial areas, in association with blood vessels and ducts (Fig. 1B). The α_1 adrenergic receptor IR observed on the acinar cells' surface appeared as small punctate labeling, whereas that in the interstitial areas appeared as larger punctate labeling (Fig. 1B). Double labeling results showed that although some of the α_1 adrenergic receptor IR appeared to colocalize

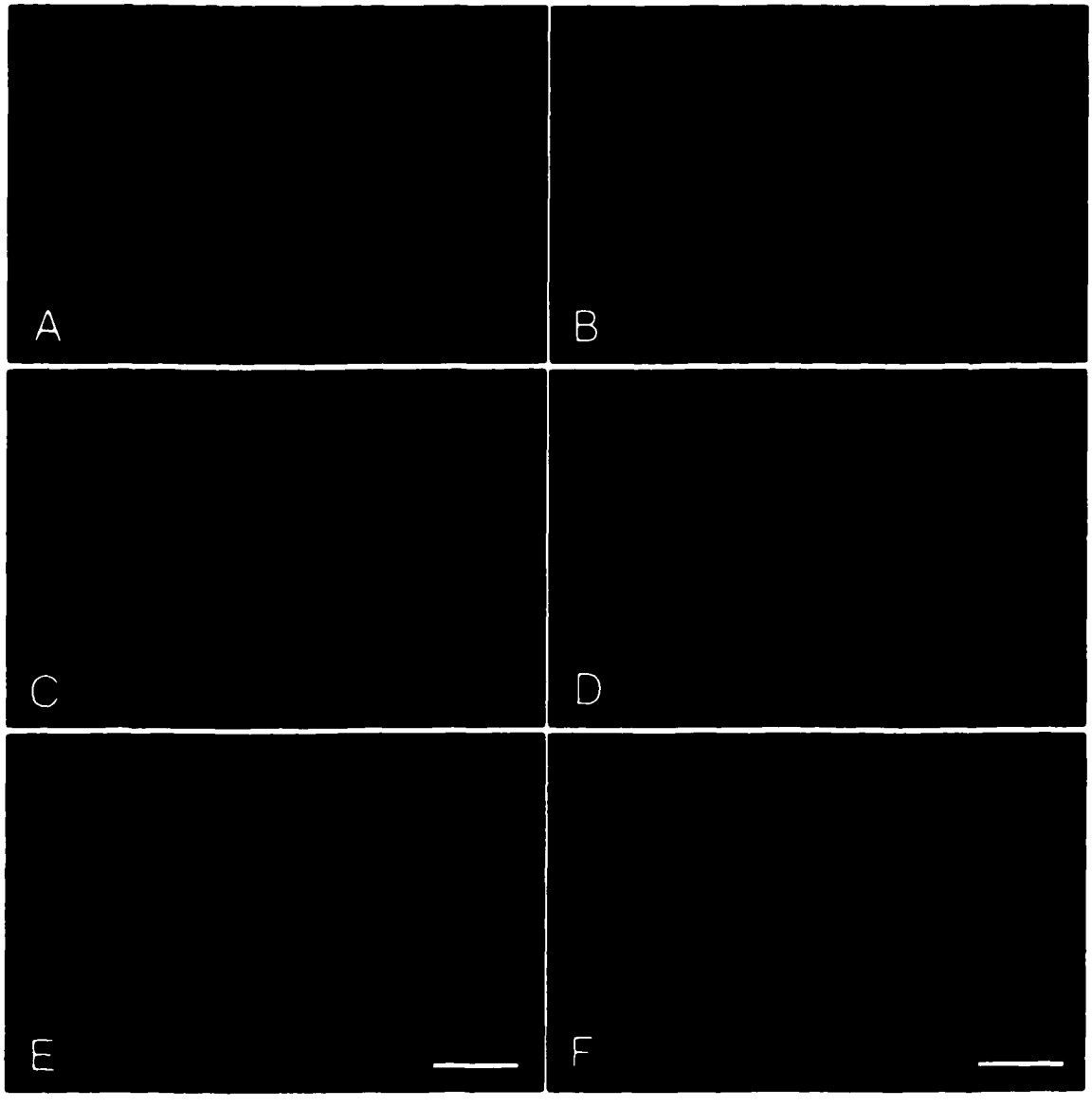

FIGURE 1. Confocal images of α_1 and β_1 adrenergic receptor immunoreactivity (IR) in the mouse lacrimal gland. **(A)** Control for α_1 adrenergic receptors. **(B)** Extensive α_1 adrenergic receptor IR was observed in the gland and it appeared that more α_1 adrenergic receptor IR was observed in association with interstitial tissue, blood vessel (arrow) and ducts. This IR appeared as larger punctate labeling compared to that in contact with the acinar cells. **(C)** At higher magnification, α_1 adrenergic receptor IR was observed on the surface and inside the acinar cells. **(D)** Control for β_1 adrenergic receptors. **(E)** Extensive β_1 adrenergic receptor IR was observed in the gland. It appeared that little was found on acinar cell surfaces and most was inside these cells. Unlike the distribution of α_1 adrenergic receptor IR, little β_1 adrenergic receptor IR could be observed in the interstitium. **(F)** α_1 adrenergic receptor IR observed at higher magnification. Note that this IR appeared to concentrate in an area within the acinar cells' cytoplasm. Scale bar in A, B, D, and E, 25 μm; in C and F, 40 μm.

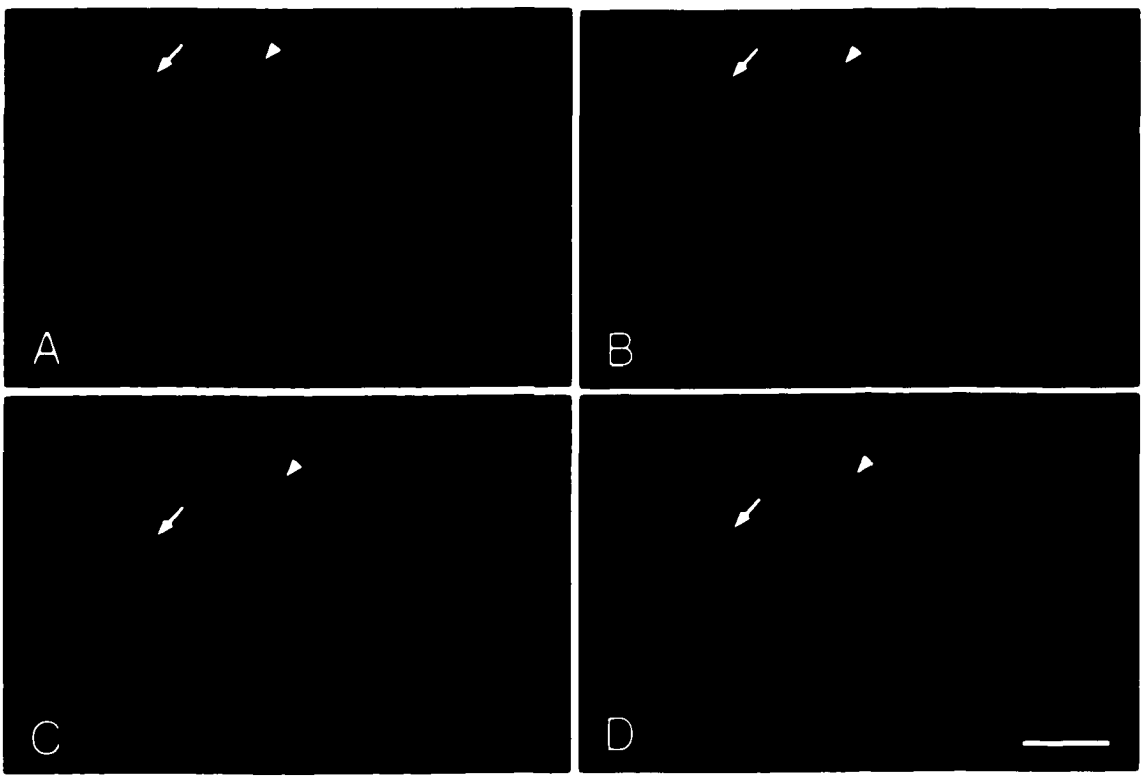

FIGURE 2. Confocal images of double labeling of α_1, β_1 adrenergic receptors and TH IR in the mouse lacrimal gland. **(A)** α_1 adrenergic receptor IR (visualized with FITC) was observed in the gland. **(B)** A few TH immunoreactive nerves (visualized with Rhodamine Red) were observed in the gland. Some of these nerves colocalized (arrow) or were in close association (arrowhead) with α_1 adrenergic receptor IR. **(C)** Extensive β_1 adrenergic receptor IR was found in the gland and it appeared that none was in close contact or colocalized with TH IR. A blood vessel (arrow) and a nerve (arrowhead) were stained with TH antibody in **(D)**. Scale bar, 40 μm.

or be in close association with TH immunoreactive nerve fibers, most was not (Figs. 2A, 2B).

Compared to the extent of α_1 adrenergic receptor IR, more β_1 adrenergic receptor IR was observed in the lacrimal gland. Some was evident on the basolateral surfaces and most appeared to be inside the acinar cells (Figs. 1E, 1F). The β_1 adrenergic receptor IR appeared to concentrate in an area within the cytoplasm of the acinar cells. Unlike the distribution of α_1 adrenergic receptor IR, almost no β_1 adrenergic receptor IR could be observed in the interstitial areas (Figs. 1E, 1F). Double-labeling results indicated that none of the β_1 adrenergic receptor IR was colocalized with TH immunoreactive nerves (Figs. 2C, 2D).

Dose Response Curves for Autonomic Agonists

Responses to norepinephrine, phenylephrine, carbachol. The dose dependency of protein secretion in response to carbachol, norepinephrine, and phenylephrine stimulation was relatively similar (Fig. 3). Protein secretion rates increased with increasing agonist concentration, and reached their maxima at 10^{-5} M, respectively. Moreover, the secretory responses for carbachol in the concentration range of 10^{-8} M to 10^{-6} M, and norepinephrine in the range of 10^{-7} M to 10^{-5} M, were almost linear. As the concentration increased further, the secretion rate reached a plateau or decreased slightly. Although carbachol, norepinephrine, and phenylephrine showed similar patterns and exhibited almost the same maximal responses, the threshold concentration of carbachol that elicited protein secretion was about 10 times lower than that of norepinephrine or phenylephrine. The half maximal concentrations (EC_{50}), the concentration at which half of the maximal

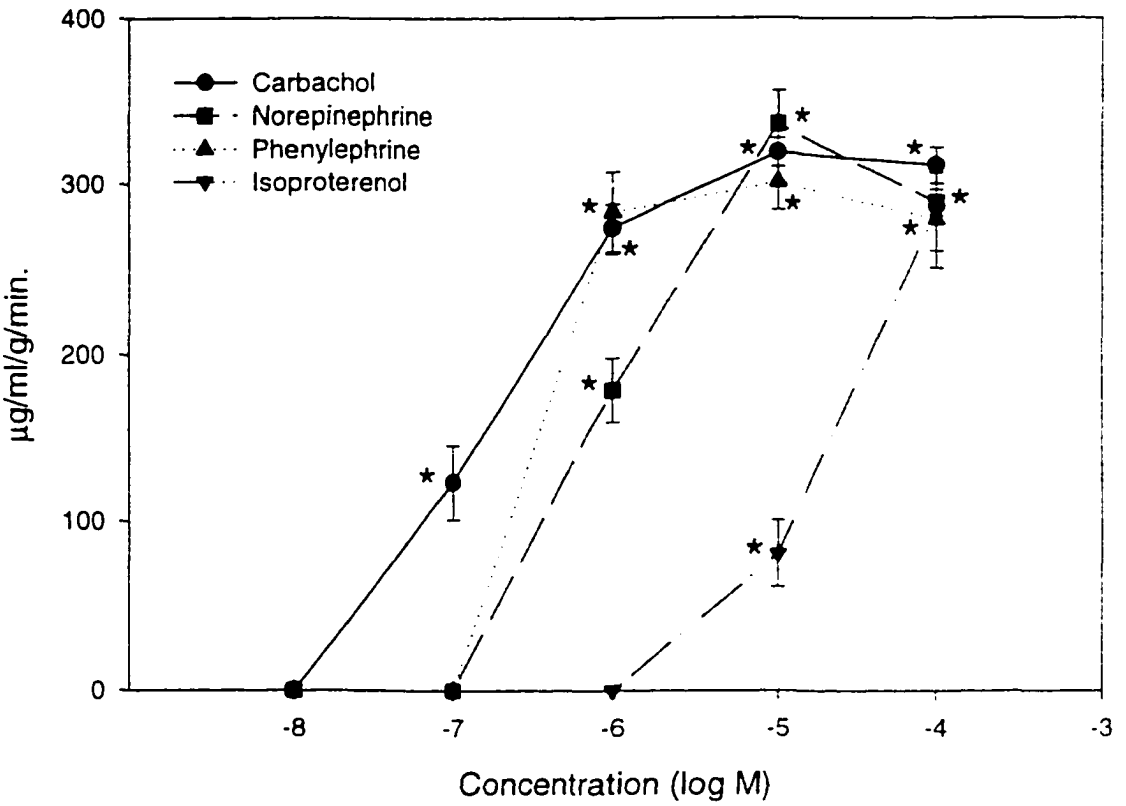

FIGURE 3. Dose response curves of protein secretion from mouse lacrimal gland fragments in response to autonomic agonists. Ordinate is total protein secretion rate (µg/ml per gram tissue per minute). Each point represents means ± SEM from 4 to 6 experiments. *Indicates significant difference from the corresponding basal secretion rate of each agonist.

response was elicited, were estimated based on the dose-response curves: carbachol (3×10^{-7} M), phenylephrine (5×10^{-7} M), and norepinephrine (10^{-6} M).

Response to isoproterenol. The protein secretion rate increased almost linearly with increasing agonist concentration. At the highest dose used (10^{-4} M), it appeared that isoproterenol had not yet achieved its maximum effect, which may be higher than that for carbachol, norepinephrine, and phenylephrine. However, the threshold for isoproterenol was much higher than the other agonists. By using the secretion rate in response to isoproterenol at 10^{-4} M as the maximal response, the EC_{50} of isoproterenol was estimated to be 3×10^{-5} M, which was about 100 times that of carbachol and 60 times that of phenylephrine.

By comparing each EC_{50}, the order of potency of these agonists can be estimated: carbachol ($EC_{50}=3\times10^{-7}$ M)>phenylephrine ($EC_{50}=5\times10^{-7}$ M) >norepinephrine ($EC_{50}=10^{-6}$ M)>isoproterenol ($EC_{50}=3\times10^{-5}$ M).

The stimulated secretion rate induced by each agonist was significantly greater ($P<0.05$) than its corresponding basal rate at all concentrations. However, at 10^{-5} M, the concentration that induced maximal secretion, no significant differences ($P>0.05$) existed among the regulated secretion rates evoked by carbachol, norepinephrine, and phenylephrine.

Protein Secretion to Agonists with the Presence of Antagonists

To further explore the specificity of the adrenergic agonists, we exposed the gland fragments to agonists at their EC_{50} in the presence of specific antagonists.

Phentolamine, a general α adrenergic receptor antagonist, inhibited phenylephrine-induced secretion in a concentration-dependent manner. At the lowest concentration used, 10^{-8} M, phentolamine caused 50% inhibition. As the concentration of phentolamine increased, secretory inhibition was also increased. A maximal inhibition of about 80% occurred at 10^{-5} M. In rat lacrimal gland acini, maximal inhibition of phenylephrine-induced peroxidase secretion by phentolamine was 90%, which was considered to be complete inhibition.[13] As the phentolamine concentration was further increased to 10^{-4} M, the inhibitory effect was slightly lower than that at 10^{-5} M (Fig. 4).

Propranolol, a general β adrenergic receptor antagonist, also inhibited protein secretion induced by isoproterenol in a concentration-dependent manner. Generally, the inhibitory effect was proportional to the concentration of propranolol. Like phentolamine, the maximal inhibition of about 52% occurred at 10^{-5} M and was significantly lower than that achieved by phentolamine on phenylephrine-induced secretion. This suggests that the inhibition by propranolol on isoproterenol-induced secretion was incomplete. As with phentolamine, the inhibitory effect of propranolol at 10^{-4} M was slightly lower than that at 10^{-5} M (Fig. 5).

Since norepinephrine can stimulate both α and β receptors and is naturally present in the body, we also used antagonists to explore the relative contribution of α and β receptor-mediated pathways. Norepinephrine was used at 10^{-6} M, the concentration that induced maximal protein secretion. Antagonists were used at 10^{-5} M, the concentration that maximally inhibited the response to the corresponding agonists. Norepinephrine and antagonists were added simultaneously. The addition of phentolamine decreased norepinephrine-induced protein secretion by about 67%, whereas the addition of

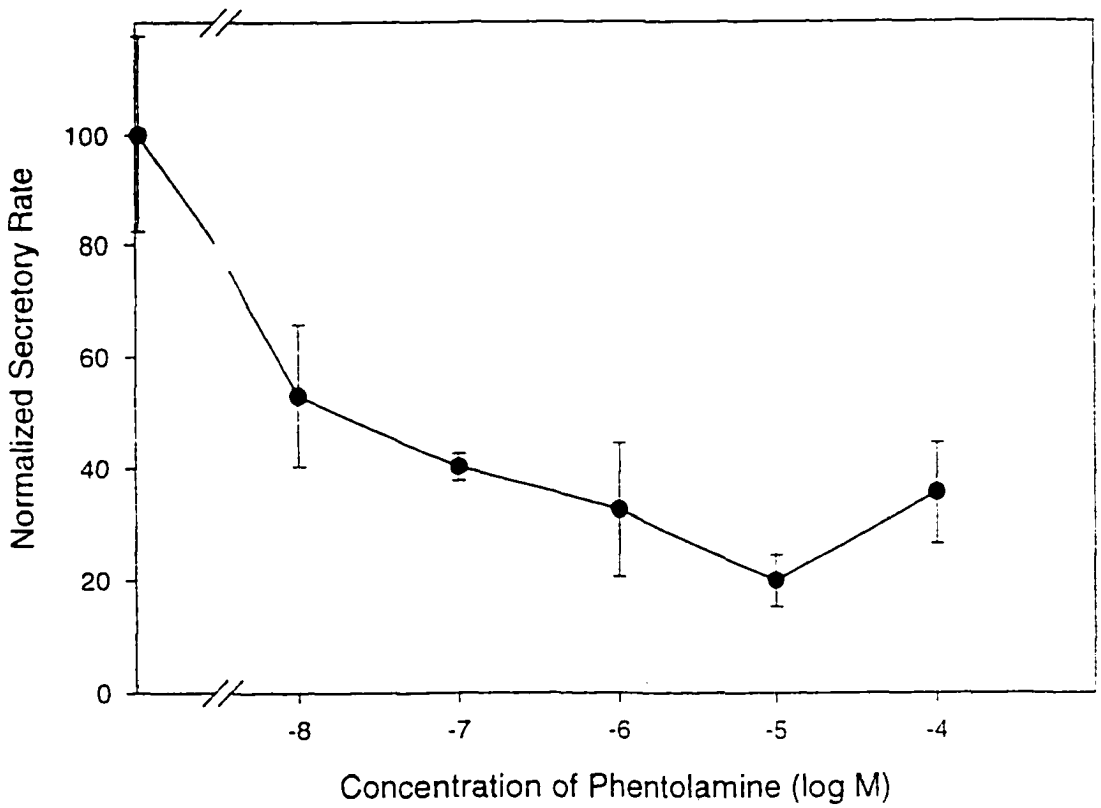

FIGURE 4. The inhibitory effects of phentolamine on protein secretion induced by phenylephrine at half maximal concentration (5×10^{-7} M). Ordinate is the percentage of inhibitory effect. Each point represents means ± SEM from 4 to 6 experiments.

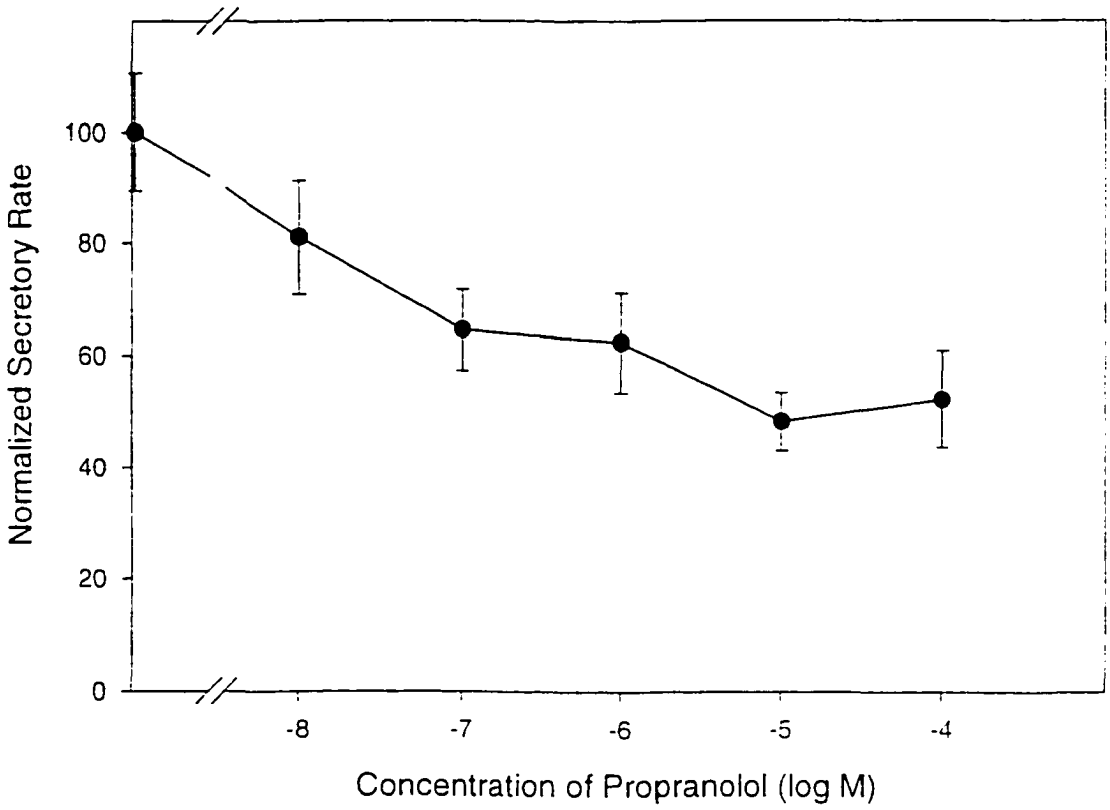

FIGURE 5. Inhibitory effect of propranolol on protein secretory responses induced by isoproterenol at half maximal concentration (3×10^{-5} M). Ordinate is the percentage of inhibitory effect. Each point represents means ± SEM from 4 to 6 experiments.

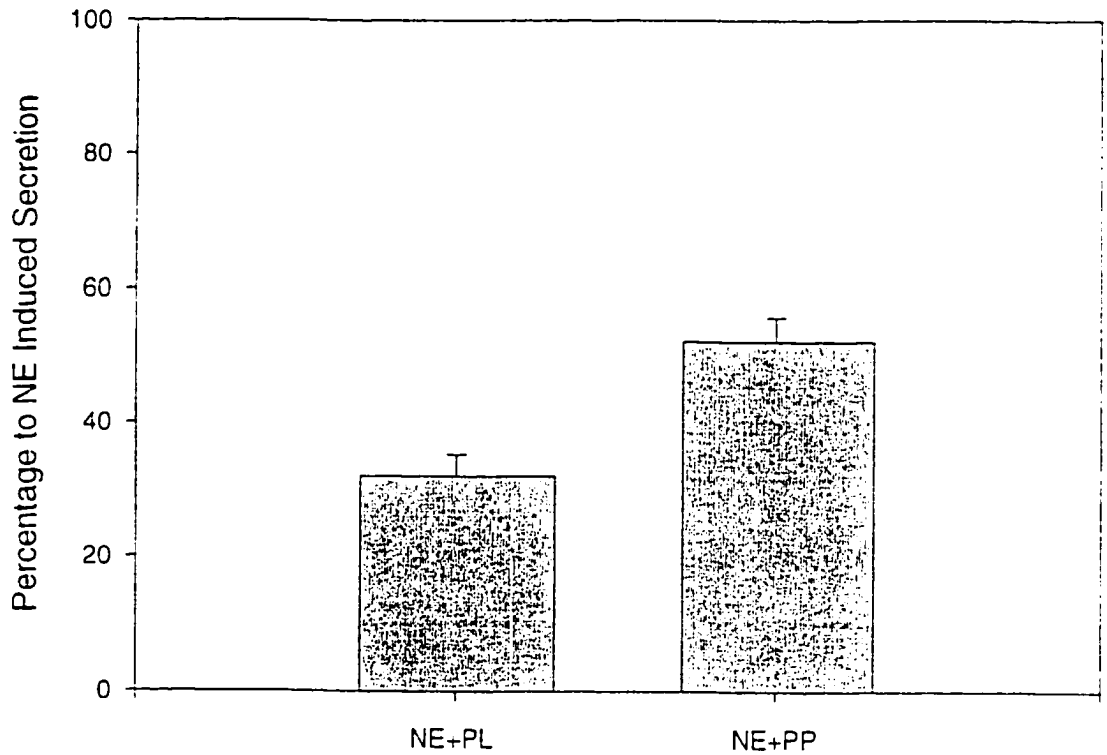

FIGURE 6. Inhibitory effects of phentolamine (PL) and propranolol (PP) on norepinephrine (NE)-induced protein secretion. Protein secretory rate induced by NE is normalized as 100 and ordinate is percentage of secretion rate with the presence of antagonists compared to it. NE (10^{-6} M). PL and PP (10^{-5} M). NE and antagonist were added simultaneously. Each point represents means ± SEM from 4 experiments.

propranalol caused a 47% decrease (Fig. 6). Neither phentolamine nor propranolol alone caused a change in the protein secretion rate.

Protein Secretion to α and β Receptors Agonists Added Together

Both agonists were used at their EC_{50}: phenylephrine (5×10^{-7} M) and isoproterenol (3×10^{-5} M). The protein secretory response to these two agonists applied simultaneously was slightly larger than that induced by either agonist alone. However, the response was much lower than the theoretical addition of the secretion elicited by phenylephrine and isoproterenol alone, which was significantly different (Fig. 7). These data suggest that there was no synergism of protein secretion between α and β adrenergic pathways.

SDS-PAGE

A previous report indicated that the individual proteins in the tear samples from llama and cattle separated out much more distinctly under non-reduced conditions.[14] However, our results showed the contrary. Better protein band separation was achieved under reduced conditions. Therefore, we used 2-mercaptoethanol throughout the experiments, and all the results presented were obtained under reduced conditions.

It appeared that there were differences between the proteins induced by phenelephrine and isoproterenol (Fig. 8). Some bands were observed in the phenylephrine-induced protein samples while they were absent in isoproterenol-induced samples, and vice versa. Even for those bands that were present in both samples, the density appeared not to be the same. The identity of these proteins is not yet known.

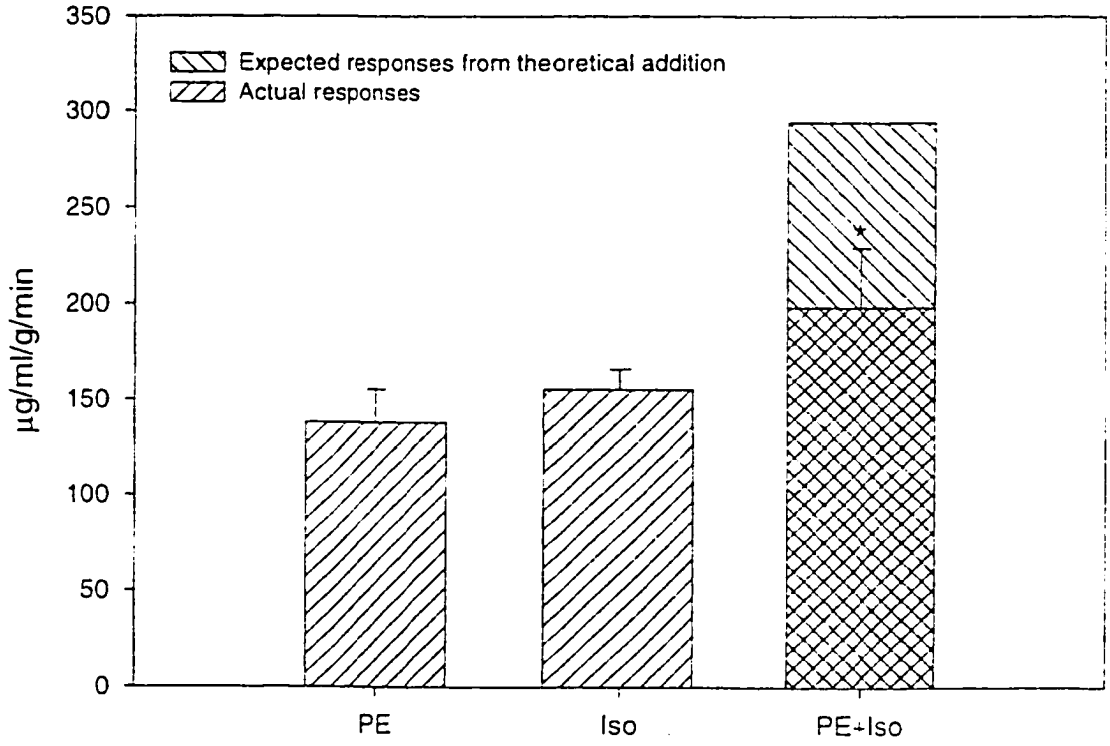

FIGURE 7. Protein secretion in response to the simultaneous addition of phenylephrine and isoproterenol showed no synergism. Agonists were used at their half maximal concentrations, phenylephrine (5×10^{-7} M), isoproterenol (3×10^{-5} M). Ordinate is the secretion rate in µg/ml per gram tissue per minute. Each point represents means ± SEM from 4 to 6 experiments. *Indicates significant difference between actual responses and theoretical addition of secretory responses to individual agonists.

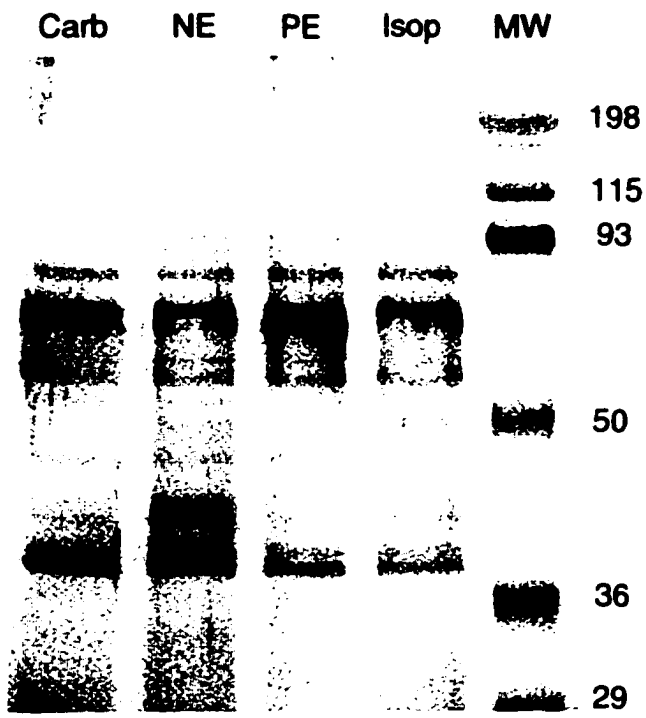

FIGURE 8. SDS-PAGE of protein samples of mouse lacrimal gland fragments induced by phenylephrine and isoproterenol. Samples were run on SDS-PAGE in 10% polyacrylamide gels. It appeared there were some differences of the protein profiles among these samples. Some bands were observed in the phenylephrine-induced protein samples while they were absent in isoproterenol-induced samples, and vice versa. Even for those bands that were present in both samples, the density appeared not to be the same.

DISCUSSION

Our confocal microscopy observations clearly showed that there was extensive α_1 and β_1 adrenergic receptor IR in the mouse lacrimal gland. To our knowledge, this is the first description of α_1 and β_1 receptor IR in the lacrimal gland. The observations suggest the presence of α_1 and β_1 adrenergic pathways in the mouse lacrimal gland and their association with lacrimal function. Protein secretion studies using adrenergic agonists were in agreement with this notion.

Norepinephrine, phenylephrine, and isoproterenol induced protein secretion from the gland fragments in a concentration-dependent manner. Protein secretion increased with the increase of agonist concentration and reached a maximum at 10^{-5} M for norepinephrine and phenylephrine and 10^{-4} M for isoproterenol. As norepinephrine, phenylephrine, and isoproterenol are competitive agonists and work through adrenergic receptors, the significant protein secretion induced by these agonists further evidenced the presence of adrenergic receptors and their direct linkage to protein secretion from the mouse lacrimal gland.

Dose-response curves obtained from norepinephrine and phenylephrine showed patterns similar to that of carbachol, the cholinergic agonist. Carbachol also induced protein secretion in a dose-dependent manner, and achieved a maximal response at 10^{-5} M. The dose response curve of carbachol in our experiments was very similar to that from rat lacrimal gland, which measured either newly synthesized protein[15] or peroxidase secretion.[16] In the concentration range of 10^{-6} M to 10^{-4} M, norepinephrine and phenylephrine induced a magnitude of protein secretion similar to that of carbachol, suggesting that the adrenergic stimulation by norepinephrine and phenylephrine was as

effective as the cholinergic activation in eliciting protein secretion. Norepinephrine and phenylephrine dose response curves were similar to those from rat lacrimal cells, which measured peroxidase secretion.[17,18]

Although carbachol, norepinephrine, and phenylephrine all induced similar amounts of protein secretion at concentrations between 10^{-6} M to 10^{-4} M, carbachol was more effective at lower concentrations ($<10^{-6}$ M). In fact, the threshold of carbachol-induced protein secretion was about 10 times lower than that for norepinephrine and phenylephrine, and at least 100 times lower than that for isoproterenol.

The dose response curve of isoproterenol was quite different from that of other agonists. At the highest dose used (10^{-4} M), it appeared that isoproterenol had not achieved its maximal response, which may be higher than that of other agonists. However, the EC_{50} of isoproterenol was about 100 times that of carbachol, and 60 times that of phenylephrine. These results are in contrast to those from rabbits, which indicated that the threshold for isoproterenol-induced protein secretion (3×10^{-8} M) was 100 times lower than that of carbachol (3×10^{-6} M).[3]

The EC_{50} of these autonomic agonists was estimated from their dose response curves, and the order of potency of these agonists was estimated as follows: carbachol (3×10^{-7} M)>phenylephrine (5×10^{-7} M) >norepinephrine (10^{-6} M)>isoproterenol (3×10^{-5} M). These results were in agreement with previous reports of the EC_{50} of carbachol, epinephrine, and phenylephrine in rats, which measured peroxidase secretion,[18-20] although one study reported an EC_{50} of phenylephrine (10^{-5} M) that was much higher than that found in the present investigation.[19]

In order to characterize the specificity of agonist-induced protein secretion, we also tested the effect of increasing concentrations of adrenergic antagonists on protein secretion induced by agonists at their EC_{50}.

Phentolamine, the non-selective α adrenergic receptor antagonist, inhibited phenylephrine-induced protein secretion in a dose-dependent manner. Even though the inhibition appeared incomplete (maximal inhibiton was 80.15% at 10^{-5} M phentolamine), protein secretion was decreased by 50% at 10^{-8} M, the lowest concentration used. In rat lacrimal gland acini, the maximal inhibition by phentolamine on phenylephrine (10^{-4} M)-induced peroxidase secretion was 90%, which was considered to be complete inhibition, whereas the half inhibition was achieved by 10^{-6} M phentolamine.[13] Another report in rats also indicated that phenylephrine-induced protein secretion was inhibited by phentolamine in a dose-dependent manner.[21] These data suggest the presence of an α_1 adrenergic receptor-mediated protein secretion pathway in the mouse lacrimal gland, which was further supported by the observation of α_1 adrenergic receptor IR in the gland.

The non-selective β adrenergic receptor antagonist, propranolol, inhibited isoproterenol-induced protein secretion in a dose-dependent manner. However, the inhibition was less specific and less effective than the inhibition by phentolamine on phenylephrine-induced secretion. Maximal inhibition of 52% was achieved at 10^{-5} M, and with 20% inhibition at 10^{-8} M. In rats, propranolol inhibits isoproterenol-induced labeled protein secretion in a dose-related manner and achieves complete inhibition.[22]

Propranolol has been reported to be a selective drug for β adrenergic receptors. These data demonstrated the presence of a β adrenergic receptor-mediated protein secretory pathway in the mouse lacrimal gland. The observation of β_1 adrenergic receptor

IR in the gland provides further support and suggests that the β adrenergic pathway may be of the β_1 type.

Both the immunofluorescence and protein secretion findings suggest there is an α_1 adrenergic pathway in the mouse lacrimal gland. Confocal microscopy demonstrated that extensive α_1 adrenergic receptor IR was present either on the surfaces or inside the cytoplasm of acinar cells, and that much more IR was found in the interstitial areas. Phenylephrine, the specific α_1 adrenergic receptor agonist, induced a significant amount of protein secretion from the gland fragments. The inhibitory effects of phentolamine on phenylephrine-induced protein secretion provide further evidence of the presence of an α adrenergic receptor-mediated protein secretory pathway. Our findings were in agreement with a previous report that α_1 adrenergic receptors were present in the mouse lacrimal gland.[23]

In the rat lacrimal gland, α adrenergic receptors were shown to be located on the acinar cells. Activation of these cells by phenylephrine could increase potassium permeability and release, and this effect was almost completely blocked by phentolamine.[4] Phenylephrine was reported to stimulate peroxidase secretion from rat lacrimocytes, and this effect was suppressed by phentolamine, even though radioligand binding assays indicated that the gland did not possess a substantial number of α_1 and α_2 adrenergic receptors.[21]

Our data also suggest the presence of a β adrenergic pathway in the mouse lacrimal gland. The dose response curve for isoproterenol indicates that although isoproterenol failed to induce protein secretion at concentrations less than 10^{-5} M, it did significantly increase protein secretion at concentrations above 10^{-5} M. The

isoproterenol-induced protein secretion increased almost linearly with the increase of agonist concentration. It appeared that isoproterenol had not yet achieved its maximal effect at 10^{-4} M, which may be higher than that of norepinephrine, phenylephrine, or carbachol. These data suggest the presence of a β adrenergic-mediated protein secretory pathway in the mouse lacrimal gland, a conclusion that is further supported by the inhibitory effect of propranolol on isoproterenol-induced response. Electrophysiological recordings from mouse lacrimal acinar cells indicated that isoproterenol markedly enhanced ATP-induced inward and outward currents, which can be blocked by propranolol.[5] These findings were in support of our results. However, some studies on mouse lacrimal gland reported data that disputes this conclusion. A pharmacological study indicated that the mouse lacrimal gland had only α_1 adrenergic receptor[23], and intracellular recordings from acinar cells failed to detect any isoproterenol-induced membrane potential and resistance change.[6]

Data supporting the β adrenergic receptor-mediated modulation of lacrimal gland function has been previously reported in the rat lacrimal gland. Isoproterenol has been shown to stimulate protein (peroxidase) secretion[22,24,25] and increase potassium permeability, even though the response could not be blocked by classical β-antagonists.[4] However, other reports claimed that isoproterenol had no effect on inducing radiolabeled protein[26] and peroxidase[19] secretions.

In rabbit, *in vivo* experiments on cannulated lacrimal gland showed that there were norepinephrine-responsive β adrenergic receptors present, which are most likely located in secretory cells. Isoproterenol was more effective than norepinephrine and

epinephrine in inducing lacrimal flow.[27] *In vitro* studies also showed the existence of β-adrenergic receptors and their association with a protein secretory response.[3]

Taken together, the present study showed three lines of evidence in support of the involvement of a β-adrenergic system in the regulation of protein secretion in the mouse lacrimal gland: 1) Isoproterenol stimulated protein secretion from the gland fragments; 2) the isoproterenol-induced secretion was specifically inhibited by propranolol, the non-selective β-adrenergic receptor antagonist; and 3) the close association of the β_1 adrenergic receptor IR with acinar cells and interstitial tissues was supported by confocal microscopy observations. The microscopy findings further suggested that the β-adrenergic pathway in the mouse lacrimal gland was of the β_1 type.

From the dose response curves, phenylephrine, at concentrations of 10^{-6} M to 10^{-4} M, appeared to have intrinsic activity similar to that of norepinephrine in eliciting protein secretion. Phenylephrine has been shown to specifically activate an α_1-adrenergic pathway to stimulate lacrimal gland protein secretion.[3] At 10^{-5} M, protein secretion induced by phenylephrine was about 90% of that induced by nerepinephrine and 97% of that at 10^{-4} M. At 10^{-6} M, phenylephrine-induced secretion was 1.58 times that from norepinephrine, which was significantly different.

The adrenergic neurotransmitter norepinephrine can activate both α and β receptors. It has been reported in rat lacrimal glands that 85% of the adrenergic regulation of labeled (newly synthesized) protein secretion was achieved through α_1 receptor activation, and the remaining 15% was assumed to be mediated by β receptor activation.[24] In order to determine the contribution of α and β adrenergic systems to norepinephrine-induced protein secretion in the mouse lacrimal gland, we added

phentolamine and propranolol, at the concentrations shown to maximally inhibit phenylephrine and isoproterenol-induced secretion, respectively, with norepinephrine at its EC_{50}. Phentolamine decreased norepinephrine-induced protein secretion by 69%, and propranolol achieved 47% inhibition. These data, together with the comparison of protein secretion rates induced by norepinephrine and phenylephrine at various concentrations, suggest that the majority of the adrenergic activation in the mouse lacrimal gland is mediated by α adrenergic receptors, and that β adrenergic receptor activation plays a relatively minor role. Our data are in rough agreement with those from rats, but stand in contrast to the data from rabbit lacrimal glands. In rabbits, phentolamine reduced norepinephrine-evoked protein secretion by 47%, whereas propranolol achieved 71% inhibition.[3]

Although both α_1 and β_1 adrenergic receptor IR were observed to be in close association with acinar cells, they exhibited different distribution patterns in the interstitial areas. It appeared that more α_1 adrenergic receptor IR was observed in the interstitial areas, in association with blood vessels and ducts, and less was in contact with acinar cells. This may suggest that the α_1 adrenergic pathway, in addition to regulating protein secretion from acinar and ductal cells, may also play a role in modulating blood flow within the gland. Vasoconstriction in the lacrimal gland has been shown to be mediated by α adrenergic receptors,[28] and in cats and rabbits, vascular tone in the lacrimal gland has been reported to be correlated with tear flow.[2,29] Other studies have also demonstrated that the blood flow in the submandibular gland influences secretory function.[30,31] In contrast, our study shows that most, if not all, of the β_1 adrenergic receptor IR is in association with acinar cells rather than with blood vessels and ducts,

suggesting that the β_1 adrenergic pathway may be involved only in the secretory process at the acinar cell level.

Even in the acinar cells, α_1 and β_1 adrenergic receptor IR appeared to have different distribution patterns. α_1 adrenergic receptor IR was observed to be relatively evenly distributed on the surfaces and inside the cytoplasm, whereas much of the β_1 adrenergic receptor IR appeared to be concentrated in the cytoplasm with only some labeling on the membrane.

Ductal cells are also responsible for secreting proteins upon activation. Since only α_1 adrenergic receptor IR was observed to be in association with ducts, and α_1 and β_1 adrenergic receptor IR exhibited different distribution patterns even at the acinar cell level, activation of α_1 and β_1 adrenergic receptors may induce secretion of different proteins. Preliminary data by SDS-PAGE indicated that there were differences between the proteins secreted in response to phenylephrine and isoproterenol (Fig. 8). This contrasts with the results obtained from rabbits, which showed no differences.[3]

When the lacrimal gland fragments were exposed to both phenylephrine and isoproterenol, the protein secretion rate was higher than that evoked by only one agonist. However, the rate was significantly lower ($P<0.05$) than the theoretical addition of the rates resulting from exposure to each agonist separately. Based on the definition of synergism,[12,24] it appears that there is no synergistic effect between the α_1 and β adrenergic activation in the mouse lacrimal gland. It has been reported that the α_1 and β adrenergic activations use different intracellular transduction pathways in the lacrimal gland. Agonist binding to the α_1 receptors partially activates the Ca^{2+}/diacylglycerol (DAG)/cGMP-dependent pathway, whereas binding to β receptors has been shown to

activate the cAMP-dependent pathway.[18,32,33] A report in rats described synergism between the α_1 and β adrenergic stimulation,[24] and in rabbits, synergism between the cholinergic and β adrenergic systems has also been reported.[3]

Although norepinephrine has been proposed to work in a "paracrine" style, its actions are mostly restricted to the extrasynaptic area because norepinephrine is inactivated quickly upon release. It seems unlikely that the norepinephrine released from the adrenergic nerve endings could diffuse across long distances and remain at physiologically significant concentrations to stimulate acinar cells. As the lacrimal gland has dense vasculature, the distance between capillary walls and acinar cells is much shorter than that between adrenergic nerve endings and distant acinar cells. Therefore, it is likely that circulating norepinephrine in the blood stream plays a role in stimulating adrenergic receptor-mediated secretion.

Although adrenergic innervation, as reflected by markers such as tyrosine hydroxylase, isn't as extensive as the parasympathetic innervation in the mouse lacrimal gland, the fact that there are systemic catecholamines circulating in the blood stream and that they can diffuse into the gland to influence secretory processes suggest that the adrenergic pathway may not necessarily be less important than the parasympathetic pathway.

It should be pointed out that we used a lacrimal gland fragment preparation in the present study, which contains acinar, ductal, myoepithelial, and endothelial cells. As acinar cells represent at least 80% of the lacrimal gland mass,[4,16] we believe the data presented here are representative of the acinar cells. However, the presence of other cell types should be considered in the interpretation of these data.

In conclusion, we have shown that there are extensive α_1 and β_1 adrenergic receptors present in the mouse lacrimal gland, and thus that there is more adrenergic innervation than previously thought. In addition, we propose that adrenergic receptors may be better a choice to reflect the extent of adrenergic-mediated pathways in the mouse lacrimal gland, in view of the catecholamines circulating in the blood stream and their possible diffusion into the lacrimal gland milieu. Adrenergic agonists induced significant protein secretion from the gland, demonstrating the adrenergic receptor-mediated protein secretory processes in the mouse lacrimal gland. The inhibitory effects of specific adrenergic antagonists on agonist-induced protein secretion provide further evidence of the existence of adrenergic pathways. Our data showed no synergism between α_1 and β receptor-mediated protein secretion. Preliminary data also suggest there are differences between proteins induced by α_1 and β adrenergic systems, which appeared to support the notion of differential secretion in the mouse lacrimal gland.

References

1. Goldstein AM, De Palau A, Botelho SY. Inhibition and facilitation of pilocarpine-induced lacrimal flow by norepinephrine. *Invest Ophthalmol.* 1967;6:498-511.

2. Botelho SY, Martinez EV, Pholpramool C, Prooyen HC, Janssen JT, De Palau A. Modification of stimulated lacrimal gland flow by sympathetic nerve impulses in rabbit. *Am J Physiol.* 1976;230:80-84.

3. Bromberg BB. Autonomic control of lacrimal protein secretion. *Invest Ophthalmol Vis Sci.* 1981;20:110-116.

4. Parod RJ, Putney JW, Jr. An alpha-adrenergic receptor mechanism controlling potassium permeability in the rat lacrimal gland acinar cell. *J Physiol.* 1978;281:359-369.

5. Sasaki T, Gallacher DV. The ATP-induced inward current in mouse lacrimal acinar cells is potentiated by isoprenaline and GTP. *J Physiol.* 1992;447:103-118.

6. Iwatsuki N, Petersen OH. Membrane potential, resistance, and intercellular communication in the lacrimal gland: effects of acetylcholine and adrenaline. *J Physiol.* 1978;275:507-520.

7. Parod RJ, Dambach GE, Putney JW Jr. Membrane potential changes in lacrimal gland acinar cells elicited by carbachol and epinephrine. *J Pharmacol Exp Ther.* 1980;213:473-479.

8. Aberg G, Adler G, Wikberg J. Inhibition and facilitation of lacrimal flow by beta-adrenergic drugs. *Acta Ophthalmol (Copenh).* 1979;57:225-235.

9. Botelho SY, Hisada M, Fuenmayor N. Functional innervation of the lacrimal gland in the cat. Origin of secretomotor fibers in the lacrimal nerve. *Arch Ophthalmol.* 1966;76:581-588.

10. Zoukhri D, Hodges RR, Dartt DA. Lacrimal gland innervation is not altered with the onset and progression of disease in a murine model of Sjögren's syndrome. *Clin Immunol Immunopathol.* 1998;89:126-133.

11. Powell CC, Martin CL. Distribution of cholinergic and adrenergic nerve fibers in the lacrimal glands of dogs. *Am J Vet Res.* 1989;50:2084-2088.

12. Dartt DA, Ronco LV, Murphy SA, Unser MF. Effect of phorbol esters on rat lacrimal gland protein secretion. *Invest Ophthalmol Vis Sci.* 1988;29:1726-1731.

13. Dartt DA, Rose PE, Dicker DM, Ronco LV, Hodges RR. Alpha 1-adrenergic agonist-stimulated protein secretion in rat exorbital lacrimal gland acini. *Exp Eye Res.* 1994;58:423-429.

14. Gionfriddo JR, Melgarejo T, Morrison EA, Alinovi CA, Asem EK, Krohne SG. Comparison of tear proteins of llamas and cattle. *Am J Vet Res.* 2000;61:1289-1293.

15. Keryer G, Rossignol B. Effect of carbachol on 45Ca uptake and protein secretion in rat lacrimal gland. *Am J Physiol.* 1976;230:99-104.

16. Herzog V, Sies H, Miller F. Exocytosis in secretory cells of rat lacrimal gland. Peroxidase release from lobules and isolated cells upon cholinergic stimulation. *J Cell Biol.* 1976;70:692-706.

17. Thorig L, Van Haeringen NJ, Timmermans PB, Van Zwieten PA. Peroxidase secretion from rat lacrimal gland cells in vitro: II. The role of calcium in stimulus-secretion coupling. *Exp Eye Res.* 1983b;37:485-492.

18. Hodges RR, Dicker DM, Rose PE, Dartt DA. Alpha 1-adrenergic and cholinergic agonists use separate signal transduction pathways in lacrimal gland. *Am J Physiol.* 1992;262:G1087-G1096.

19. Putney JW Jr, VandeWalle CM, Leslie BA. Stimulus-secretion coupling in the rat lacrimal gland. *Am J Physiol.* 1978;235:C188-C198.

20. Dartt DA, Baker AK, Vaillant C, Rose PE. Vasoactive intestinal polypeptide stimulation of protein secretion from rat lacrimal gland acini. *Am J Physiol.* 1984;247:G502-G509.

21. Thorig L, Van Haeringen NJ, Timmermans PB, Van Zwieten PA. Peroxidase secretion from rat lacrimal gland cells in vitro. I. Alpha-adrenergic stimulation in the absence of alpha-adrenoceptors. *Exp Eye Res.* 1983a;37:475-483.

22. Mauduit P, Herman G, Rossignol B. Protein secretion induced by isoproterenol or pentoxifylline in lacrimal gland: Ca2+ effects. *Am J Physiol.* 1984;246:C37-C44.

23. Qi JH, Li BH. Effects of alpha adrenoceptor agonists and antagonists on palpebral fissure and lacrimation in mice. *Zhongguo Yao Li Xue Bao.* 1992;13:153-156.

24. Mauduit P, Herman G, Rossignol B. Protein secretion in lacrimal gland: alpha 1-beta-adrenergic synergism. *Am J Physiol.* 1986;250:C704-C712.

25. Friedman ZY, Lowe M, Selinger Z. beta-Adrenergic receptors stimulated peroxidase secretion from rat lacrimal gland. *Biochim Biophys Acta.* 1981;675:40-45.

26. Jahn R, Padel U, Porsch PH, Soling HD. Adrenocorticotropic hormone and alpha-melanocyte-stimulating hormone induce secretion and protein phosphorylation in the rat lacrimal gland by activation of a cAMP-dependent pathway. *Eur J Biochem.* 1982;126:623-629.

27. Botelho SY, Goldstein AM, Martinez EV. Norepinephrine-responsive beta-adrenergic receptors in rabbit lacrimal gland. *Am J Physiol.* 1973;224:1119-1122.

28. Granstam E, Nilsson SF. Non-adrenergic sympathetic vasoconstriction in the eye and some other facial tissues in the rabbit. *Eur J Pharmacol.* 1990;175:175-186.

29. Yasui T, Karita K, Izumi H, Tamai M. Correlation between vasodilatation and secretion in the lacrimal gland elicited by stimulation of the cornea and facial nerve root of the cat. *Invest Ophthalmol Vis Sci.* 1997;38:2476-2482.

30. Hanna SJ, Brelen ME, Edwards AV. Effects of reducing submandibular blood flow on secretory responses to parasympathetic stimulation in anaesthetized cats. *Exp Physiol.* 1999;84:677-687.

31. Rourke K, Edwards AV. Submandibular secretory and vascular responses to stimulation of the parasympathetic innervation in anesthetized cats. *J Appl Physiol.* 2000;89:1964-1970.

32. Dartt DA. Signal transduction and control of lacrimal gland protein secretion: a review. *Curr Eye Res.* 1989;8:619-636.

33. Dartt DA, Hodges RR, Zoukhri D. Signal transduction pathways activated by cholinergic and alpha 1-adrenergic agonists in the lacrimal gland. *Adv Exp Med Biol.* 1998;438:113-121.

NEURONAL NITRIC OXIDE SYNTHASE AND THE AUTONOMIC INNERVATION OF THE MOUSE LACRIMAL GLAND

by

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ABSTRACT

PURPOSE. To determine the expression patterns of the vesicular acetylcholine transporter (VAChT), tyrosine hydroxylase (TH), and neuronal nitric oxide synthase (nNOS) in the pterygopalatine ganglion (PPG) and the lacrimal gland of normal mice.

METHODS. Mouse PPG and lacrimal glands were processed for single- and double- labeled indirect immunofluorescence studies. Slides were examined with conventional fluorescence microscopy and confocal laser scanning microscopy.

RESULTS. All the somata in the PPG expressed both VAChT and nNOS immunoreactivity (IR). The postganglionic axons within the ganglion showed less VAChT-IR intensity than that seen in the somata, whereas nNOS-IR was almost undetectable. In the lacrimal gland, nNOS-positive nerve bundles and fibers were observed to be associated with tear-collecting ducts, blood vessels, and acini. Some nNOS-positive punctate elements appeared to be distributed among acini. Many nerve fibers were VAChT-immunoreactive and a small number of fibers were TH immunoreactive in the gland. Most of the VAChT-positive fibers, and some of the TH-positive nerves displayed nNOS-IR.

CONCLUSIONS. The expression of nNOS in cells of the PPG and in lacrimal gland nerves suggests that nitric oxide (NO) may play a role in modulating tear production. The site of action may include the PPG, ducts, blood vessels, acini, nerve fibers, and myoepithelial cells within the gland. NO may modulate parasympathetic and/or sympathetic synaptic transmission or by acting directly on lacrimal gland components. The interaction between NO-ergic and the conventional autonomic input illustrates the complexity of the innervation pattern of the mouse lacrimal gland.

INTRODUCTION

The mammalian lacrimal gland has dense parasympathetic innervation[1-3] and, in some species, sympathetic innervation.[4-7] Most of the parasympathetic nerve fibers that innervate the lacrimal gland arise from neurons in the pterygopalatine ganglion (PPG).[1-3] The PPG is the parasympathetic ganglion that receives synaptic input from preganglionic parasympathetic neurons in the brain stem. Postganglionic nerve fibers from the PPG innervate the nasal and palatine mucosae, the extra- and intracranial vasculature, and the lacrimal gland.[8]

Nitric oxide (NO) is a diffusible neurotransmitter that mediates a variety of physiological functions. NO is synthesized by nitric oxide synthase (NOS), which has three isoforms: neuronal NOS (nNOS), epithelial NOS (eNOS), and inducible NOS (iNOS).[9] Previous studies have reported that nNOS immunoreactivity (IR) is expressed in the PPG and nasal mucosae of rats,[9] and humans.[10] Because the lacrimal gland receives dense parasympathetic innervation and some sympathetic innervation in some species, we speculated that nNOS may also exist in the lacrimal gland and that NO might play a role in the neural control of its function. In light of the various physiological functions that NO mediates in the central and peripheral nervous systems and its potential role in the regulation of lacrimal gland secretion, we set out to investigate the expression patterns of nNOS in the normal mouse PPG and lacrimal gland and its relationship to the autonomic innervation.

Choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) are the enzymes that are responsible for the synthesis and breakdown of acetylcholine (ACh), and both ChAT and AChE have been used in attempts to visualize the distribution of

cholinergic fibers in tissues. However, there are reports that AChE, as revealed by conventional histochemical and immunohistochemical detection methods, is not restricted to cholinergic neurons[11] and that the synthesis of ChAT is not necessarily restricted to cholinergic neurons.[12] These reports suggest that neither AChE nor ChAT is an unambiguous marker for cholinergic neurons.

The vesicular acetylcholine transporter (VAChT) is a proton-dependent transporter that is responsible for packaging ACh into synaptic vesicles, and the immunohistochemical detection of VAChT has emerged as a reliable method for the detection of cholinergic neurons.[13] Using a direct double-labeling method in rat, Arvidsson et al.[13] found VAChT and ChAT colocalized in neurons and suggested VAChT as a novel and reliable marker in the autonomic nervous system for cholinergic neurons, which innervate organs such as the salivary and lacrimal glands. Other reports showed that VAChT IR is more sensitive than most ChAT antibodies for the detection of cholinergic terminals[14] and provides a clearer signal than ChAT labeling.[14,15] All these results suggest that VAChT is a highly specific marker for cholinergic somata and nerve fibers. Moreover, although VAChT is present in both the neurons' somata and terminals, it is concentrated in the terminals and thus better reflects the distribution of cholinergic synapses.[15]

Tyrosine hydroxylase (TH) is the rate-limiting enzyme responsible for the synthesis of dopamine and has been used as the marker for the sympathetic innervation of the lacrimal gland. Therefore, we have used antisera to nNOS, VAChT, and TH to examine the distribution of these markers in the pterygopalatine ganglion and lacrimal gland of the mouse. Our results have confirmed that all the neurons in the PPG are

positive for VAChT as well as nNOS. Most of the nerve fibers in the glands were positive for VAChT, with many also showing nNOS IR. Some TH-positive fibers were seen in the gland and a few of them were also positive for nNOS. These data suggest that NO could play a role in the regulation of lacrimal gland secretion.

MATERIALS AND METHODS

Tissue Preparation

Five Swiss Webster (SW) and five C57 female mice were obtained from commercial vendors (Taconic Farms or Charles River). All animals were kept in a 12-hour light-dark cycle and maintained in an accredited animal facility with freely available food and water. They were managed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To obtain tissues, the animals were killed with an overdose of halothane and decapitated around midday. The lacrimal glands and PPG were removed and placed in freshly prepared 4% buffered paraformaldehyde. After 3 to 4 hours of fixation at 4 °C, the tissue was placed in 0.1 M phosphate buffer containing 30% sucrose at pH 7.4 for at least 12 hours at 4 °C. The lacrimal gland and ganglia were then placed in optimal cutting temperature (OCT) embedding medium (Sakura Finetek USA, Torrance, CA), serially sectioned at 10 μm with a cryostat (Leica, Deerfield, IL), and collected on slides (Superfrost Plus; VWR Scientific, West Chester, PA). The sections were dried and stored at –20 °C until used.

Immunohistochemistry

The antibodies used were polyclonal rabbit anti-rat NOS1 (R-20; Santa Cruz Biotechnology, Santa Cruz, CA), at dilutions of 1:400 for lacrimal gland sections and 1:200 for ganglion; goat anti-VAChT polyclonal antibody (Chemicon International, Temecula, CA) at dilutions of 1:2,000 for lacrimal gland, 1:1,000 for ganglion. The VAChT antibody has been well characterized and has been shown to colocalize with ChAT in both central and peripheral nervous systems, including lacrimal gland.[13,14] The sheep anti-TH polyclonal antibody (Chemicon) was used at a dilution of 1:200. Sections were incubated in primary antibody diluted with 0.1 M sodium phosphate buffer (PBS) overnight or for 48 hours for TH. For control samples, primary antibodies were omitted. Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit and anti-sheep IgG, and Texas Red-conjugated donkey anti-goat and anti-sheep IgG (Jackson Immunoresearch, West Grove, PA), all at a dilution of 1:200. The secondary antibodies were applied for 1 hour. The slides were then washed with three changes of PBS and one change of 4 mM sodium carbonate (pH 10.0), cover-slipped, and examined with a conventional fluorescence microscope (Eclipse E800M; Nikon, Melville, NY). The images were captured with a digital camera (Spot; Diagnostics Instruments, Sterling Heights, MI), and analyzed on a desktop computer with image analysis software (PhotoShop; Adobe Systems, Mountain View, CA). Additional images were also obtained with a confocal laser scanning microscope (TCS SP; Leica). At least 21 sections were examined per gland to examine all lobes within the gland.

RESULTS

Pterygopalatine Ganglion

In the PPG, all the neurons appeared to display various intensities of nNOS IR. The neurons, which were interspersed among nerve fibers that do not synapse within the PPG, were of various sizes (20-40 μm) and were clearly differentiated from the nerve fibers (Fig. 1A). nNOS IR appeared to be distributed relatively evenly within the neuronal somata, except that labeling was excluded from the nucleus (Fig. 1B). However, nNOS-IR was weak or undetectable in the axons of these cells in contrast to the easily observed fibers in the lacrimal gland, which is one of the targets of the neurons in the PPG.

All the neurons in the PPG also displayed VAChT-IR. The somata were of different sizes and, except for the nucleus, were filled by the labeling (Fig. 1C). VAChT-IR was also detectable in presynaptic terminals that resembled large baskets. Axons were clearly visible as well but were less intensely labeled. The labeling of both the cell bodies and the axons stood out clearly from the rest of the nerve fibers that course through the ganglion without synapsing. nNOS and VAChT appeared to be colocalized in all of the neuronal somata in the PPG.

Lacrimal Gland

Many nerve fibers in the lacrimal gland displayed nNOS IR, and there were smaller bundles of fibers with numerous varicosities that could be observed around tear-collecting ducts, blood vessels, and acini.

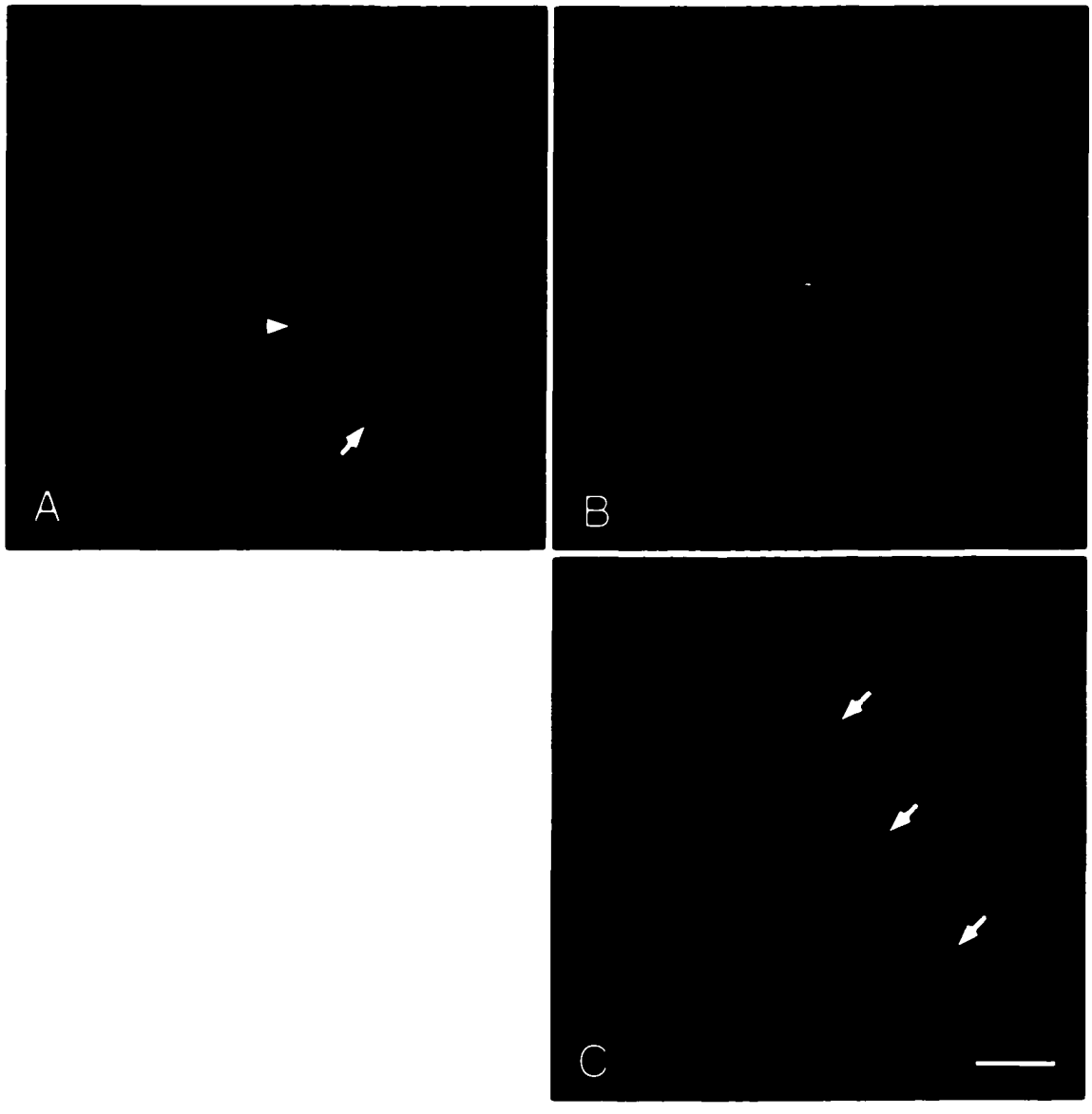

FIGURE 1. VAChT and nNOS labeling in the PPG. **(A)** VAChT visualized with FITC was observed in the somata, with presynaptic terminals shown as large puncta (arrow), and VAChT-IR in the postganglionic somata appears uniformly throughout the cytoplasm with some small puncta visible (arrowhead). **(B)** nNOS-FITC was expressed in all the somata, but virtually no nNOS-IR could be detected in the axons. **(C)** VAChT-Texas Red was detected in both somata and axons, though the intensity in axons appeared weaker than in the somata (arrows). In the somata, nNOS-IR and VAChT-IR was colocalized. **(B)** and **(C)** are from the confocal microscope. Scale bar, 30 μm.

Large bundles of nerve fibers were labeled, mostly in the interlobular areas, running along with large tear-collecting ducts and blood vessels. Smaller nerve bundles and fibers were observed to branch from the larger ones. Some nerve fibers were observed to be closely associated with collecting ducts, and some appeared to encircle the outer surface of ducts (Fig. 2A). Some punctate nNOS IR could also be observed in association with acinar and ductal cells. Some nerve fibers with prominent varicosities appeared to be in close proximity to the acini (Figs. 2A, 2B, 2E). Although quantification was not performed in the present study, examination of serial sections indicated that approximately 80% to 90% of the acini exhibited either punctate nNOS-immunoreactive staining or were in close contact with nNOS-positive nerve fibers.

Dense VAChT-IR was found in the lacrimal gland. Numerous varicosities were observed among acini, presumably representing synaptic vesicles containing ACh (Fig. 2C). These VAChT-positive varicosities were close to the basolateral membrane of acini. These varicosities formed a loose network around the acini, and each nerve fiber appeared to be in close association with more than one acinar cell.

Some nerve fibers were also TH immunoreactive. However, compared with the dense VAChT-positive varicosities in the gland, many fewer TH-positive varicosities were observed. Most of the TH IR was seen in association with the blood vessels, which are known to be innervated by sympathetic neurons (Fig. 2D). Some of the labeled nerves were observed to course among acini, and nerve fibers with many varicosities were occasionally seen in close association with acini (Fig. 2F).

nNOS IR was colocalized in many but not all of the fibers that displayed VAChT IR (Figs. 2B, 2C). Also, some nNOS-positive fibers that did not display VAChT IR were

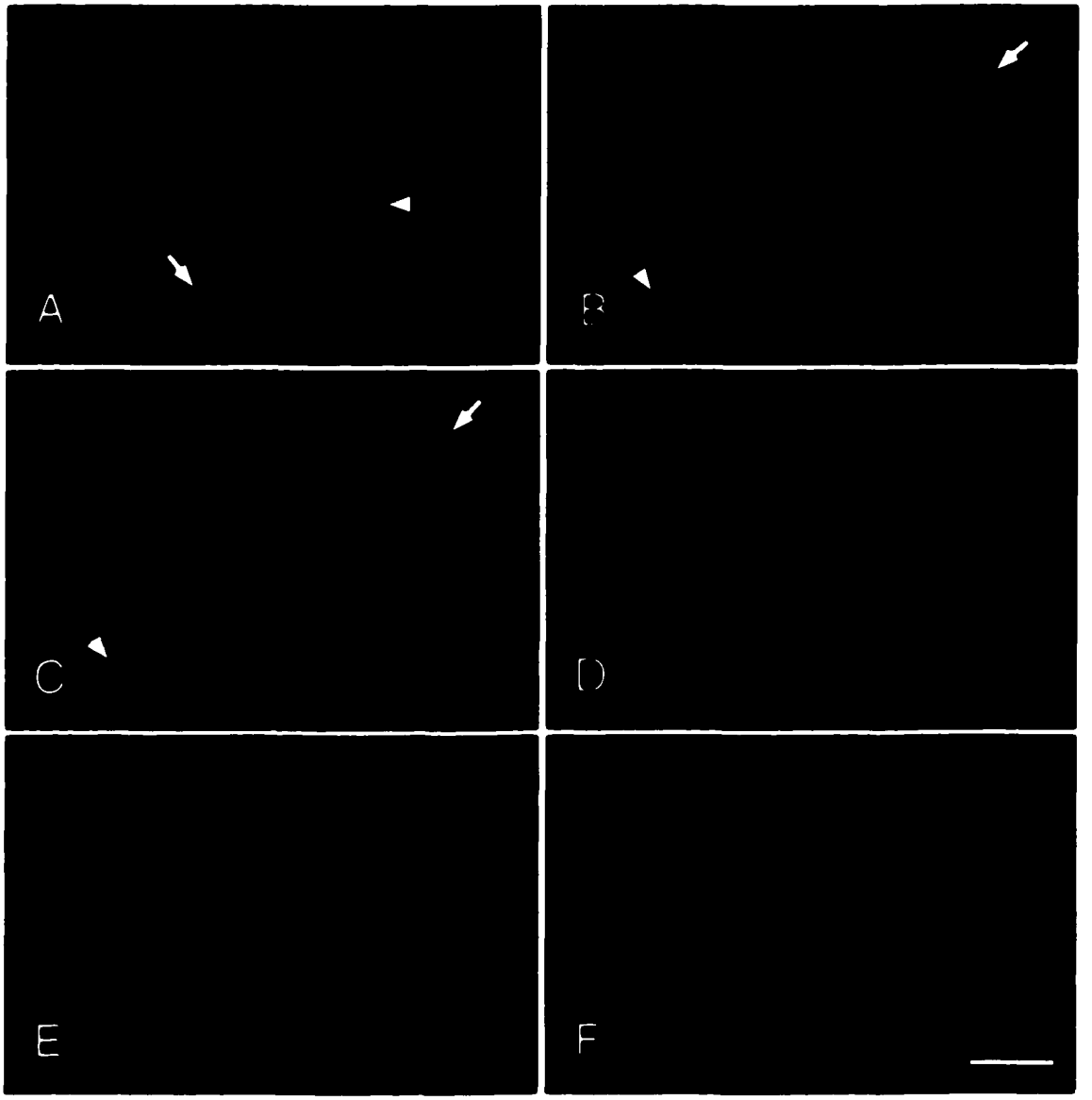

FIGURE 2. nNOS, VAChT, and TH IR in the lacrimal gland. (**A**) Nerve bundles (arrow) and single fibers, were observed to be immunoreactive to nNOS. Some nerve fibers with varicosities were in close proximity with acini. A single nerve fiber was observed to encircle the duct (arrowhead). Some punctate nNOS labeling was found in both acinar and ductal cells. (**B**) Many nerve fibers (arrow) were nNOS immunoreactive. Extensive punctate labeling was also observed in the cytoplasm of acinar cells (arrowhead). (**C**) Dense fibers with numerous varicosities (arrow) and punctate labeling (arrowhead), which were close to the basolateral side of acini, showed VAChT IR. Many VAChT IR colocalized with nNOS IR. (**D**) Nerve fibers innervating the blood vessels were observed to be immunoreactive to TH, with typical meshwork-like labeling. (**E, F**) A single nerve fiber that was nNOS immunoreactive (**E**) also displayed TH IR (**F**). While these labels appeared to be colocalized, we cannot rule out the possibility that the labeling represents two different fibers. (**B**) and (**C**) are from the confocal microscope. Scale bar, 30 μm.

observed. However, in most of the fibers nNOS-IR appeared to colocalize with VAChT IR. nNOS IR was detectable in some, but not all, of the sparse TH-positive fibers that coursed among the acini. However, no nNOS IR was observed to colocalize with TH-IR in the inner layer of blood vessel walls.

DISCUSSION

The PPG is a parasympathetic relay center and receives its inputs from the preganglionic parasympathetic neurons in the brainstem through the greater superficial petrosal nerve. These preganglionic fibers then synapse with the neurons in the ganglion and the postganglionic axons project to target organs, such as the lacrimal gland and nasal mucosae.

The present study demonstrated that virtually all the neurons in the mouse PPG were VAChT immunoreactive. To our knowledge, this is the first description of VAChT IR in the neurons of the PPG in mouse. Our results are consistent with those of other investigators who used ChAT or AChE as the cholinergic marker and found that virtually all the PPG neurons in rat,[16] chicken,[17] and pigeon[18] display ChAT or AChE IR. Also, the present study demonstrated that most of the neurons in the PPG were nNOS immunoreactive. This is in agreement with other reports in monkey,[19] human,[9,19] mouse,[20] rat,[10] pigeon,[18] and cat.[21] The neuronal profiles in the mouse PPG varied in size and labeling intensity for both VAChT and nNOS. However, there was no apparent systematic relationship between the neurons' somata size and the observed labeling intensity. This result was consistent with the observations in the human PPG.[9]

An interesting observation in the present study was the apparent difference in the labeling pattern of VAChT and nNOS in the portion of the axons within PPG (Figs. 1B, 1C). In the case of VAChT IR, even though the labeling in the somata was more intense than that in axons, the axons also displayed clear VAChT IR. However, nNOS IR was virtually undetectable in the axons, even though the somata were clearly nNOS positive. This observation was in contrast to reports in pigeon,[18] and rat,[10] which have demonstrated that the postganglionic axons in the PPG were also nNOS immunoreactive. Because most of the parasympathetic nerves in the lacrimal gland originate from the PPG and many of them were immunoreactive to nNOS, and because all the neurons in the PPG showed both VAChT and nNOS immunoreactivity, it would be reasonable to expect nNOS to be detectable in the axons that extend from the neurons to the lacrimal gland. However, it is possible that NO synthesized in the postganglionic somata serves to modulate synaptic transmission within the ganglion and that in the terminals in the gland it modulates secretion. That nNOS was undetectable in the portions of the axons within the PPG may indicate that nNOS, which is being transported to the terminal, is present in quantities below the detection threshold for the methods used.

The presence of NOS in the preganglionic[22] and postganglionic PPG neurons suggests that NO may play a role in neurotransmission within the PPG. Retrograde labeling studies of the preganglionic parasympathetic neurons projecting to the PPG in rabbit demonstrated that while all of the retrogradely labeled neurons in the brain stem displayed ChAT IR, only 75% were NOS positive, although the NOS isoform was not specified.[22] Our observations further demonstrated nNOS in the postganglionic neurons within PPG colocalized with VAChT in all the neurons' somata. Studies in *Torpedo*

synaptosomes indicated that NO may decrease ACh release,[23] and other reports have shown that NO can act as a neuromodulator in modulating synaptic transmission at several types of synapses.[24-27] There are thus two potential sources of NO that might play a role in modulating cholinergic transmission within the PPG: the preganglionic neuronal terminals and the postganglionic somata.

Although there are reports of NOS distribution in the PPG and some of its targets, such as nasal mucosae,[10,20] sinus mucosa,[28] and cranial blood vessels,[21] to our knowledge, this is the first report of nNOS-IR in postganglionic neurons of the PPG and nerve fibers in the lacrimal gland.

It has been established that the mammalian lacrimal gland is densely innervated by the parasympathetic nervous system.[1,2,29] Our observations, based on VAChT as a parasympathetic marker, confirmed that the mouse lacrimal gland was densely innervated by VAChT-positive nerve fibers. The small nerve bundles and fibers that were VAChT immunoreactive appeared to be found only at the base of the acinar cells. They were never seen to project into the acini or between acinar cells. These observations were consistent with findings in cat.[4] The VAChT-positive nerve fibers included large and small nerve bundles that coursed along with tear-collecting ducts and apparent nerve fibers that appeared to form a dense network. Every acinar cell seemed to be in close proximity to a VAChT-positive nerve fiber.

In contrast to the dense distribution of parasympathetic nerves in the lacrimal gland, only sparse TH IR nerve fibers were detectable in the gland among the secretory acini, although more TH-IR was present in the inner layer of interlobular blood vessels. The sympathetic innervation of the lacrimal gland varies among species, and there are

some discrepancies between the observations of different investigators. For example, there are reports that every acinar cell is in close proximity to an adrenergic fiber in cat,[4] dog,[5,7] human, and monkey.[6] However, there are reports of findings in other studies in humans[30] and in monkeys[31] that are inconsistent with these findings. In rat[7,32] and mouse,[7] only very sparse TH-positive nerve fibers have been described, and most of them were in association with the blood vessels, whereas only a few were found among acini. In guinea pig, some researchers reported rich adrenergic innervation in lacrimal glands,[32] whereas others described a virtual absence in the gland except along the blood vessels.[7] Our observations appeared to be in agreement with a previous report in the mouse.[7]

Many of the VAChT-positive, and some of the TH-positive, nerve fibers in the lacrimal gland also appeared to exhibit nNOS IR. Although the role of NO in the lacrimal gland is not known, the colocalizations of nNOS with VAChT and with TH, in addition to the highly diffusive nature of NO, support the notion that there might be some interaction between NO and other autonomic transmitter systems within the lacrimal gland. For example, as discussed with respect to the PPG, NO could exert its effects by influencing ACh transmission.

The observation of TH-immunoreactive nerve fibers coursing among acini suggests that sympathetic nerves may play a direct role in modulating tear secretion, in addition to regulating blood flow within the lacrimal gland. The presence of nNOS IR in some of the TH-immunoreactive fibers raises the possibility that there may be some interaction between these two systems as well.

There are five targets that could be influenced by NO at the lacrimal gland level: ducts, blood vessels, acini, nerve fibers, and myoepithelial cells. Many of the nNOS-

positive nerve bundles ran alongside the ducts in the interlobular areas, some of the smaller nerve fibers and their varicosities appeared to be in close association with the ducts (Fig. 2A). Physiologically, NO is a potent vasoactive agent[33-35] and can readily diffuse across membranes and mediate vasodilation within vascular smooth muscle.[36-38] Thus, the blood vessels in the lacrimal gland, which are primarily innervated by sympathetic nerves and supplemented by some parasympathetic ones,[39] might be modulated by NO. This is significant, because in the cat and rabbit,vasodilation in the lacrimal gland was correlated with tear flow.[40,41] Other studies have also demonstrated that the blood flow in the submandibular gland of cats could influence secretory function.[42,43]

Ultrastructural studies have demonstrated that nerve terminals in the monkey lacrimal gland are in close proximity to acinar cells.[44] In the present study, numerous parasympathetic and fewer sympathetic fibers were observed among the acini, and many of the parasympathetic and some sympathetic fibers exhibited nNOS labeling. It is possible, therefore, that NO may influence tear production from the acini, which are the functional units of the lacrimal gland. Based on the fact that most nerve fibers have nNOS and that NO is a highly diffusive molecule and can readily spread to adjacent areas, it is reasonable to speculate that NO could spread and exert its influence on most or perhaps all of the acini.

Although little is known about the functions of myoepithelial cells, it has been suggested that they could be involved in the release of secretory product by contraction. However, others have proposed that these cells' only role is to maintain the contour of the glandular end pieces, serving as the exoskeleton of the acini.[45] It has been shown that

these cells are attached to the acini in a lacy arrangement, that they contain a network of α-smooth muscle actin which is similar to that of smooth muscle,[46,47] and that M_3 muscarinic receptors are expressed on their surfaces.[47] In that NO has been reported to influence cholinergic neurotransmission[23] and muscular tone,[36-38] it can be speculated that NO may also exert some influence on the myoepithelial cells. The exact mechanism and to what extent these cells are involved are unknown.

Functional studies in the salivary glands, which have some similarities to lacrimal glands anatomically and functionally, have shown that NO can modulate protein, electrolytes, and water production in salivary secretion.[48-52] NO may influence tear secretion through various mechanisms, including control of cGMP production,[36-38,53] modulation of ion channels,[54,55] activation of intracellular signaling pathways,[56] and regulation of Na^+, K^+-ATPase.[57]

It must be emphasized that it is still unclear what role NO plays in the lacrimal gland, although research on salivary glands has shown that NO plays a role in salivary secretion. Another point that should be made is that because our observations were made in the course of a study of Sjögren's syndrome, we used only female mice, and the findings reported here may not be applicable to male mice.

In summary, nNOS was expressed in both the neurons in the mouse PPG and nerve fibers in the lacrimal gland. In the PPG, nNOS was present in all the VAChT-positive neurons, and many of the parasympathetic and some sympathetic nerves in the lacrimal gland colocalized with the nNOS-positive nerves. These observations support the notion that NO may play a role in modulating tear production through various mechanisms. These observations illustrate the complexity of both the innervation pattern

of the mouse lacrimal gland and of the mechanisms by which autonomic input may influence the secretory process.

References

1. Botelho SY, Hisada M, Fuenmayor N. Functional innervation of the lacrimal gland in the cat. Origin of secretomotor fibers in the lacrimal nerve. *Arch Ophthalmol.* 1966;76:581-588.

2. Ruskell GL. Changes in nerve terminals and acini of the lacrimal gland and changes in secretion induced by autonomic denervation. *Z Zellforsch Mikrosk Anat.* 1969;94:261-281.

3. Ruskell GL. The distribution of autonomic post-ganglionic nerve fibers to the lacrimal gland in monkeys. *J Anat.* 1971;109:229-242.

4. Ehinger B. Distribution of adrenergic nerves in the eye and some related structures in the cat. *Acta Physiol Scand.* 1966;66:123-128.

5. Powell CC, Martin CL. Distribution of cholinergic and adrenergic nerve fibers in the lacrimal glands of dogs. *Am J Vet Res.* 1989;50:2084-2088.

6. Ehinger B. Adrenergic nerves to the eye and to related structures in man and in the cynomolgus monkey (Macaca irus). *Invest Ophthalmol.* 1966;5:42-52.

7. Ehinger B. Ocular and orbital vegetative nerves. *Acta Physiol Scand Suppl.* 1966;268:1-35.

8. Hara H, Zhang QJ, Kuroyanagi T, Kobayashi S. Parasympathetic cerebrovascular innervation: an anterograde tracing from the sphenopalatine ganglion in the rat. *Neurosurgery.* 1993;32:822-827.

9. Lee SH, Iwanaga T, Hoshi O, Adachi I, Fujita T. Nitric oxide synthase in rat nasal mucosa; Immunohistochemical and histochemical localization. *Acta Otolaryngol.* 1995;115:823-829.

10. Hanazawa T, Tanaka K, Chiba T, Konno A. Distribution and origin of nitric oxide synthase-containing nerve fibers in human nasal mucosa. *Acta Otolaryngol.* 1997;117:735-737.

11. Wu D, Hersh LB. Choline acetyltransferase: Celebrating its fiftieth year. *J Neurochem.* 1994;62:1653-1663.

12. Ibanez CF, Pelto-Huikko M, Soder O, et al. Expression of choline acetyltransferase mRNA in spermatogenic cells results in an accumulation of the enzyme in the postacrosomal region of mature spermatozoa. *Proc Natl Acad Sci USA.* 1991;88:3676-3680.

13. Arvidsson U, Riedl M, Elde R, Meister. Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. *J Comp Neurol.* 1997;378:454-467.

14. Gilmor ML, Nash NR, Roghani A, et al. Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. *J Neurosci.* 1996;16:2179-2190.

15. Yao W, Godfrey DA. Vesicular acetylcholine transporter in the rat cochlear nucleus: an imunohistochemical study. *J Histochem Cytochem.* 1999;47:83-90.

16. Mione MC, Sancesario G, D'Angelo V, Bernardi G. Increase of dopamine beta-hydroxylase immunoreactivity in non-noradrenergic nerves of rat cerebral arteries following long-term sympathectomy. *Neurosci Lett.* 1991;123:167-171.

17. Walcott B, Sibony PA, Keyser KT. Neuropeptides and the innervation of the avian lacrimal gland. *Invest Ophthalmol Vis Sci.* 1989;30:1666-1674.

18. Cuthbertson S, Jackson B, Toledo C, et al. Innervation of orbital and choroidal blood vessels by the pterygopalatine ganglion in pigeons. *J Comp Neurol.* 1997;386:422-442.

19. Demer JL, Poukens V, Miller JM, Micevych P. Innervation of extraocular pulley smooth muscle in monkeys and humans. *Invest Ophthalmol Vis Sci.* 1997;38:1774-1785.

20. Matsuda H, Kusakabe T, Kawakami T, Takenaka T, Sawada H, Tsukuda M. Coexistence of nitric oxide synthase and neuropeptides in the mouse vomeronasal organ demonstrated by a combination of double immunofluorescence labeling and a multiple dye filter. *Brain Res.* 1996;712:35-39.

21. Goadsby PJ, Uddman R, Edvinsson L. Cerebral vasodilatation in the cat involves nitric oxide from parasympathetic nerves. *Brain Res.* 1996;707:110-118.

22. Zhu BS, Gibbins IL, Blessing WW. Preganglionic parasympathetic neurons projecting to the sphenopalatine ganglion contain nitric oxide synthase in the rabbit. *Brain Res.* 1997;769:168-172.

23. Ribera J, Marsal J, Casanovas A, Hukkanen M, Tarabal O, Esquerda JE. Nitric oxide synthase in rat neuromuscular junctions and in nerve terminals of *Torpedo* electric organ: its role as regulator of acetylcholine release. *J Neurosci Res.* 1998;51:90-102.

24. Shibuki K, Okada D. Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. *Nature.* 1991;349:326-328.

25. Haley JE, Wilcox GL, Chapman PF. The role of nitric oxide in hippocampal long-term potentiation. *Neuron.* 1992;8:211-216.

26. Quinson N, Miolan JP, Niel JP. Muscarinic receptor activation is a prerequisite for the endogenous release of nitric oxide modulating nicotinic transmission within the coeliac ganglion in the rabbit. *Neuroscience.* 2000;95:1129-1138.

27. Yamada K, Kushiku K, Yamada H, et al. Contribution of nitric oxide to the presynaptic inhibition by endothelin ETB receptor of the canine stellate ganglionic transmission. *J Pharmacol Exp Ther.* 1999;290:1175-1181.

28. Runer T, Cervin A, Lindberg S, Uddman R. Nitric oxide is a regulator of mucociliary activity in the upper respiratory tract. *Otolaryngol Head Neck Surg.* 1998;119:278-287.

29. De Haas EBH. Lacrimal gland response to parasympathomimetics after parasympathetic denervation. *Arch Ophthalmol.* 1960;64:34-43.

30. Seifert P, Stuppi S, Spitznas M, Weihe E. Differential distribution of neuronal markers and neuropeptides in the human lacrimal gland. *Graefe's Arch Clin Exp Ophthalmol.* 1996;234:232-240.

31. Matsumoto Y, Tanabe T, Ueda S. Kawata M. Immunohistochemical and enzymehistochemical studies of peptidergic, aminergic and cholinergic innervation of the lacrimal gland of the monkey (Macaca fuscata). *J Auton Nerv Syst.* 1992;37:207-214.

32. Nikkinen A, Uusitalo H, Lehtosalo JI, Palkama A. Distribution of adrenergic nerves in the lacrimal glands of guinea-pig and rat. *Exp Eye Res.* 1985;40:751-756.

33. Ayajiki K, Okamura T, Toda N. Nitric oxide mediates, and acetylcholine modulates, neurally induced relaxation of bovine cerebral arteries. *Neuroscience.* 1993;54:819-825.

34. Faraci FM. Role of nitric oxide in regulation of basilar artery tone in vivo. *Am J Physiol.* 1990;259:H1216-1221.

35. Gonzalez C, Estrada C. Nitric oxide mediates the neurogenic vasodilation of bovine cerebral arteries. *J Cereb Blood Flow Metab.* 1991;11:366-370.

36. Kukovetz WR, Holzmann S, Wurm A, Poch G. Evidence for cyclic GMP-mediated relaxant effects of nitro-compounds in coronary smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol.* 1979;310:129-138.

37. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and veins is nitric oxide. *Proc Natl Acad Sci USA.* 1987;84:9265-9269.

38. Mollace V, Salvemini D, Anggard E, Vane J. Nitric oxide from vascular smooth muscle cells: regulation of platelet reactivity and smooth muscle cell guanylate cyclase. *Br J Pharmacol.* 1991;104:633-638.

39. Ruskell GL. Vasomotor axons of the lacrimal glands of monkeys and the ultrastructural identification of sympathetic terminals. *Z Zellforsch Mikrosk Anat.* 1967;83:321-333.

40. Botelho SY, Martinez EV, Pholpramool C, Prooyen HC, Janssen JT, De Palau A. Modification of stimulated lacrimal gland flow by sympathetic nerve impulses in rabbit. *Am J Physiol.* 1976;230:80-84.

41. Yasui T, Karita K, Izumi H, Tamai M. Correlation between vasodilatation and secretion in the lacrimal gland elicited by stimulation of the cornea and facial nerve root of the cat. *Invest Ophthalmol Vis Sci.* 1997;38:2476-2482.

42. Rourke K, Edwards AV. Submandibular secretory and vascular responses to stimulation of the parasympathetic innervation in anesthetized cats. *J Appl Physiol.* 2000;89:1964-1970.

43. Hanna SJ, Brelen ME, Edwards AV. Effects of reducing submandibular blood flow on secretory responses to parasympathetic stimulation in anaesthetized cats. *Exp Physiol.* 1999;84:677-687.

44. Ruskell GL. The fine structure of nerve terminations in the lacrimal glands of monkeys. *J Anat.* 1968;103:65-76.

45. Satoh Y, Oomori Y, Ishikawa K, Ono K. Configuration of myoepithelial cells in various exocrine glands of guinea pigs. *Anat Embryol.* 1994;189:227-236.

46. Hodges RR, Zoukhri D, Sergheraert C, Zieske JD, Dartt DA. Identification of vasoactive intestinal peptide receptor subtypes in the lacrimal gland and their signal-transducing components. *Invest Ophthalmol Vis Res.* 1997;38:610-619.

47. Lemullois M, Rossignol B, Mauduit P. Immunolocalization of myoepithelial cells in isolated acini of rat exorbital lacrimal gland: cellular distribution of muscarinic receptors. *Biol Cell.* 1996;86:175-181.

48. Takai N, Uchihashi K, Higuchi K, Yoshida Y, Yamaguchi M. Localization of neuronal-constitutive nitric oxide synthase and secretory regulation by nitric oxide in the rat submandibular and sublingual glands. *Arch Oral Bio.* 1999;44:745-750.

49. Buckle AD, Parker SJ, Bloom SR, Edwards, AV. The role of nitric oxide in the control of protein secretion in the submandibular gland of the cat. *Exp Physiol.* 1995;80:1019-1030.

50. Edwards AV, Tobin G, Ekstrom J, Bloom SR. Nitric oxide and release of the peptide VIP from parasympathetic terminals in the submandibular gland of the anaesthetized cat. *Exp Physiol.* 1996;81:349-359.

51. Tobin G, Edwards AV, Bloom SR, Ekstrom J. Nitric oxide in the control of submandibular salivary gland function in the anaesthetized ferret. *Exp Physiol.* 1997;82:825-836.

52. Hanna SJ, Edwards AV. The role of nitric oxide in the control of protein secretion in the parotid gland of anaesthetized sheep. *Exp Physiol.* 1998;83:533-544.

53. Knowles RG, Palacios M, Palmer RM, Moncada S. Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc Natl Acad Sci USA.* 1989;86:5159-5162.

54. Light DB, Corbin JD, Stanton BA. Dual ion-channel regulation by cyclic GMP and cyclic GMP-dependent protein kinase. *Nature.* 1990;344:336-339.

55. Kaupp UB. The cyclic nucleotide-gated channels of vertebrate photoreceptors and olfactory epithelium. *Trends Neurosci.* 1991;14:150-157.

56. Rapoport RM, Draznin MB, Murad F. Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature.* 1983;306:174-176.

57. Sato T, Kamata Y, Irifune M, Nishikawa T. Inhibitory effect of several nitric oxide-generating compounds on purified Na^+, K^+-ATPase activity from porcine cerebral cortex. *J Neurochem.* 1997;68:1312-1318.

SUMMARY

Adequate tear secretion of appropriate composition is critical for maintaining the health of the cornea and conjunctiva. The exorbital lacrimal gland is the major source of tear secretion in mammals. Tear secretion is highly regulated and controlled by such factors as hormones and neural activity. In the mammalian exorbital lacrimal gland, neural control plays a major role in regulating tear secretory process.

Decreased tear secretion may cause dry eye syndrome, characterized by eye irritation, corneal damage and, in many instances, impaired vision.[110] One type of dry eye is associated with Sjögren's Syndrome (SS). SS is a chronic autoimmune disease that affects excretory glands, such as salivary and lacrimal glands, and sometimes extra-glandular organs (primary SS). Some patients may also have other connective tissue diseases, most commonly rheumatoid arthritis (secondary SS). SS is the second most common connective tissue disease, with a prevalence of about 1-2% of the general population, with women over middle-age comprising of 80-90% of the affected individuals.[110] In the US alone, the disease afflicts 2-4 million Americans, and many go undiagnosed. One of the most common symptoms is dry eye and dry mouth, and since SS is incurable, most of the management methods today are to relieve these symptoms by artificial tears that have given rise to a pharmaceutical market in the US of over $100 million per year.[110]

SS is a leading cause of aqueous tear-deficient dry eye. Unfortunately, the cause of SS is unclear.[111,112] In SS, the lacrimal gland fails to secrete tear fluid in response to

reflex stimulation, even though there is apparently healthy tissue in the glands.[113] This suggests that the neural control of the gland may be compromised. Our research on the neural control mechanisms of the mouse lacrimal gland may help us to better understand the gland's innervation pattern and its functional relevance in both normal and pathological conditions.

In the first part of the dissertation (first paper), we found that there are regional differences of innervation density and distribution pattern in the mouse lacrimal gland. By using both immunohistochemical and sucrose-potassium phosphate-glyoxylic acid (SPG) techniques, we found that the gland can be divided into two different areas based on the innervation density and distribution pattern. One area, about 10-30% of the whole gland, has much higher innervation density than the rest of the gland. The majority of the innervation in the high-ID area appeared to be of sympathetic origin, whereas most of the innervation in the low-ID area was of parasympathetic origin. The regional differences of innervation density is reminiscent of the innervation pattern in the avian Harderian gland. The innervation density of the Harderian gland is highly uneven, with much denser innervation observed in the medulla than the cortex. Adrenergic agonists induced a similar magnitude of protein secretion to that of the cholinergic agonist. At the concentration of 10^{-5} M, norepinephrine (which activates both α and β receptors) and phenylephrine (an α_1 receptor agonist) induced an increase in protein secretion similar to those induced by the cholinergic agonist carbachol. Isoproterenol, a β adrenergic receptor agonist, also stimulated protein secretion, though it was less effective than norepinephrine, phenylephrine, or carbachol. These data suggest that there is much more extensive adrenergic innervation in the mouse lacrimal gland than previously thought and

that it plays a direct and significant role in inducing protein secretion. The division of the mouse lacrimal gland into two areas suggests that the mouse lacrimal gland might be a mixed gland and that these two areas may play different roles in secreting tears of different composition in different situations. Our data support the notion that differential secretion is accomplished by activating different populations of secretory cells.

In the second part (second paper), we described the distribution of α_1 and β_1 adrenergic receptor (AR) immunoreactivity in the mouse lacrimal gland by confocal microscopy. We also explored the dose dependency of protein secretion induced by norepinephrine, phenylephrine, isoproterenol, and carbachol, at concentrations ranging from 10^{-8} M to 10^{-4} M. To further investigate the specificity of the responses to these autonomic agonists, we also studied protein secretion in response to the application of phenylephrine and isoproterenol at their half-maximal concentrations, with or without their corresponding antagonists (phentolamine for α AR and propranolol for β AR). These functional studies demonstrated that direct stimulation of either α_1 or β AR can induce significant protein secretion from the lacrimal gland. The specificity of this stimulation was further explored by the effects of specific antagonists. Our results once again support the notion that there is significant adrenergic control of the protein secretory process in the mouse lacrimal gland. In addition to norepinephrine produced and released by the adrenergic nerves present in the lacrimal glands, there is norepinephrine circulating in the blood stream. Therefore, the adrenergic receptors may be better markers to reflect the extent of adrenergic control. Traditional markers of adrenergic innervation, such as tyrosine hydroxylase (TH) or dopamine β-hydroxylase (DBH), may underestimate the extent and magnitude of adrenergic control, as they only

reflect the local sources of norepinephrine. Our data showed no synergism between α_1 and β receptor-mediated protein secretions. Preliminary data also suggest there are differences between proteins induced by α_1 and β adrenergic systems, which appeared to support the notion of differential secretion in the mouse lacrimal gland.

In the third part of the dissertation (third paper), we described the detection of nNOS IR in the neurons in PPG and lacrimal gland, by both confocal and conventional microscopy. We found that virtually all the neurons' somata in the PPG expressed both VAChT and nNOS IR. The postganglionic axons within the ganglion showed less VAChT IR intensity than that seen in the somata, while the nNOS IR was almost undetectable. In the lacrimal gland, nNOS-positive nerve bundles and fibers were observed to be associated with tear collecting ducts, blood vessels, and acini. Some nNOS-positive punctate elements also appeared to be distributed among acini. Many nerve fibers were VAChT-immunoreactive, and a smaller number of fibers were TH-immunoreactive in the gland. Double-labeling observations indicated that most of the VAChT-positive fibers, and some of the TH-positive nerves, also displayed nNOS IR. These findings suggest that NO may be produced in nerve fibers in the gland and play a role in modulating tear production, although the exact mechanism is unclear. NO may modulate parasympathetic and/or sympathetic synaptic transmission or act directly on lacrimal gland components. Candidate sites of action may include the synapses within PPG, tear collecting ducts, blood vessels, acini, nerve fibers, and myoepithelial cells within the gland. The detection of nNOS, as an indicator of NO, in the lacrimal gland suggests that NO may play a role in lacrimal gland function. The potential interaction

between the NO-ergic and the conventional autonomic innervation illustrates the complexity of the neural control in the mouse lacrimal gland.

In summary, the data presented here support the following conclusions

1. There is extensive sympathetic innervation of the mouse lacrimal gland, and it plays a direct and significant role in modulating protein secretion.

2. The mouse lacrimal gland can be divided into two distinct areas, based on the innervation density. These observations are consistent with the possibility that differential secretion might be accomplished by activating different populations of secretory cells.

3. Adrenergic receptors may be better markers for evaluating the true extent of adrenergic control than markers such as TH and DBH. The diffusion of norepinephrine circulating in the blood steam into the lacrimal gland may play a more significant role than that produced locally. Therefore, using either TH or DBH as the marker may underestimate the extent of adrenergic influence on lacrimal gland function.

4. nNOS IR is expressed in the neurons in PPG and autonomic nerves in the lacrimal gland. nNOS IR also colocalizes with most parasympathetic and some sympathetic system markers. These data suggest that NO may play a role in modulating tear secretion.

Implications and Future Studies

1. For the protein secretion studies in the present dissertation, we used total protein assay as the index. Sympathetic and parasympathetic agonists activate different intracellular transduction pathways, and the proteins secreted in response to stimulation

by specific agonists may be different. Therefore, studies assaying individual proteins are needed to determine their secretory changes.

2. Immunohistochemical observations clearly indicate that nNOS is present in neurons in PPG and in the lacrimal gland, and that nNOS IR also colocalizes with the IR of autonomic markers, suggesting that NO may play a role in lacrimal function. Functional studies are required to provide unambiguous evidence that NO plays a role in modulating tear secretion.

3. Our preliminary immunohistochemistry results indicated that more nNOS IR was found in older NZB/W mice, an animal model of Sjögren's Syndrome, suggesting that nNOS activity was upregulated and that NO may be overproduced in these animals. We hypothesize that NO may play a role in the onset and progression of Sjögren's Syndrome. Several lines of evidence from other studies support this idea.[72-74,76,99] Sjögren's Syndrome is usually characterized by lymphocyte infiltration in the salivary and lacrimal glands and subsequent damage to their functions. The available data indicate that NO might be a potential mediator of T-cell responses[52,57,58,101,102] Sjögren's Syndrome patients have elevated concentration of NO_2^-, one of the end products of NO, in their saliva.[66] More detailed studies, both physiological and biochemical, are needed to further elucidate the possible role NO may play in SS.

LIST OF REFERENCES

1. Hodges RR, Zoukhri D, Sergheraert C, Zieske JD, Dartt DA. Identification of vasoactive intestinal peptide receptor subtypes in the lacrimal gland and their signal-transducing components. *Invest Ophthalmol Vis Res.* 1997;38:610-619.

2. Lemullois M, Rossignol B, Mauduit P. Immunolocalization of myoepithelial cells in isolated acini of rat exorbital lacrimal gland: cellular distribution of muscarinic receptors. *Biol Cell.* 1996;86:175-181.

3. Satoh Y, Oomori Y, Ishikawa K, Ono K. Configuration of myoepithelial cells in various exocrine glands of guinea pigs. *Anat Embryol.* 1994;189:227-236.

4. Fullard RJ, Snyder C. Protein levels in nonstimulated and stimulated tears of normal human subjects. *Invest Ophthalmol Vis Sci.* 1990;31:1119-1126.

5. Botelho SY, Hisada M, Fuenmayor N. Functional innervation of the lacrimal gland in the cat. Origin of secretomotor fibers in the lacrimal nerve. *Arch Ophthalmol.* 1966;76:581-588.

6. Ruskell GL. Changes in nerve terminals and acini of the lacrimal gland and changes in secretion induced by autonomic denervation. *Z Zellforsch Mikrosk Anat.* 1969;94:261-281.

7. Ruskell GL. The distribution of autonomic post-ganglionic nerve fibers to the lacrimal gland in monkeys. *J Anat.* 1971;109:229-242.

8. De Haas EBH. Lacrimal gland response to parasympathomimetics after parasympathetic denervation. *Arch Ophthalmol.* 1960;64:34-43.

9. Ruskell GL. Vasomotor axons of the lacrimal glands of monkeys and the ultrastructural identification of sympathetic terminals. *Z Zellforsch Mikrosk Anat.* 1967;83:321-333.

10. Ruskell GL. The fine structure of nerve termination in the lacrimal glands of monkeys. *J Anat.* 1968;103:65-76.

11. Ehinger B. Distribution of adrenergic nerves in the eye and some related structures in the cat. *Acta Physiol Scand.* 1966;66:123-128.

12. Ehinger B. Adrenergic nerves to the eye and to related structures in man and in the cynomolgus monkey (Macaca irus). *Invest Ophthalmol.* 1966;5:42-52.

13. Ehinger B. Ocular and orbital vegetative nerves. *Acta Physiol Scand.* 1966;67(Suppl):5-35.

14. Powell CC, Martin CL. Distribution of cholinergic and adrenergic nerve fibers in the lacrimal glands of dogs. *Am J Vet Res.* 1989;50:2084-2088.

15. Lundberg JM, Anggard A, Fahrenkrug J, Hokfelt T, Mutt V. Vasoactive intestinal polypeptide in cholinergic neurons of exocrine glands: Functional significance of coexisting transmitters for vasodilatation and secretion. *Proc Natl Acad Sci. USA.* 1980;77:1651-1655.

16. Lundberg JM. Evidence for coexistence of vasoactive intestinal polypeptide (VIP) and acetylcholine in neurons of cat exocrine glands. *Acta Physiol Scand Suppl.* 1981;496:1-57.

17. Lundberg JM, Anggard A, Fahrenkrug J, Lundgren G, Holmstedt B. Corelease of VIP and acetylcholine in relation to blood flow and salivary secretion in cat submandibular salivary gland. *Acta Physiol Scand.* 1982;115:525-528.

18. Johansson O, Lundberg JM. Ultrastructural localisation of VIP-like immunoreactivity in large dense-core vesicles of 'cholinergic-type' nerve terminals in cat exocrine glands. *Neuroscience.* 1981;5:847-862.

19. Dartt DA, Baker AK, Vaillant C, et al. Vasoactive intestinal polypeptide stimulation of protein secretion from rat lacrimal gland acini. *Am J Physiol.* 1984;247:G502-G509.

20. Bromberg BB. Autonomic control of lacrimal protein secretion. *Invest Ophthalmol Vis Sci.* 1981;20:110-116.

21. Walcott B, Cameron RH, Brink PR. The anatomy and innervation of lacrimal glands. *Adv Exp Med Bio.* 1994;350:11-18.

22. Botelho SY, Hisada M, Fuenmayor N. Functional innervation of the lacrimal gland in the cat. Origin of secretomotor fibers in the lacrimal nerve. *Arch Ophthalmol.* 1966;76:581-588.

23. Ruskell GL. Changes in nerve terminals and acini of the lacrimal gland and changes in secretion induced by autonomic denervation. *Z Zellforsch Mikrosk Anat.* 1969;94:261-281.

24. Ruskell GL. The distribution of autonomic post-ganglionic nerve fibers to the lacrimal gland in monkeys. *J Anat.* 1971;109:229-242.

25. Mione MC, Sancesario G, D'Angelo V, Bernardi G. Increase of dopamine β-hydroxylase immunoreactivity in non-noradrenergic nerves of rat cerebral arteries following long-term sympathectomy. *Neurosci Lett.* 1991;123:167-171.

26. Cuthbertson S, Jackson B, Toledo C, et al. Innervation of orbital and choroidal blood vessels by the pterygopalatine ganglion in pigeons. *J Comp Neurol.* 1997;386:422-442.

27. Hara H, Zhang QJ, Kuroyanagi T, et al. Parasympathetic cerebrovascular innervation: an anterograde tracing from the sphenopalatine ganglion in the rat. *Neurosurgery.* 1993;32:822-827.

28. Wu D, Hersh LB. Choline acetyltransferase: Celebrating its fiftieth year. *J Neurochem.* 1994;62:1653-1663.

29. Arvidsson ULF, Riedl M, Elde R, et al. Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. *J Comp Neurol.* 1997;378:454-467.

30. Gilmore ML, Nash NR, Roghani A, et al. Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. *J Neurosci.* 1996;16:2179-2190.

31. Ibanez CF, Pelto-Huikko M, Soder O, et al. Expression of choline acetyltransferase mRNA in spermatogenic cells results in an accumulation of the enzyme in the postacrosomal region of mature spermatozoa. *Proc Natl Acad Sci. USA.* 1991;88:3676-3680.

32. Yao W, Godfrey DA. Vesicular acetylcholine transporter in the rat cochlear nucleus: an imunohistochemical study. *J Histochem Cytochem.* 1999;47:83-90.

33. Mauduit P, Herman G, Rossignol B. Newly synthesized protein secretion in rat lacrimal gland: post-second messenger synergism. *Am J Physiol.* 1987;253:C514-524.

34. Bacman S, Perez Leiros C, Sterin-Borda L, Hubscher O, Arana R, Borda E. Autoantibodies against lacrimal gland M3 muscarinic acetylcholine receptors in patients with primary Sjogren's syndrome. *Invest Ophthalmol Vis Sci.* 1998;39:151-156.

35. Oyster CW. The human eye: structure and function. Sunderland, MA: Sinauer Associates; 1999:228-230.

36. Botelho SY. Martinez EV. Pholpramool C. Prooyen HC. Janssen JT. De Palau A. Modification of stimulated lacrimal gland flow by sympathetic nerve impulses in rabbit. *Am J Physiol.* 1976;230:80-84.

37. Hanna SJ, Brelen ME, Edwards AV. Effects of reducing submandibular blood flow on secretory responses to parasympathetic stimulation in anaesthetized cats. *Exp Physiol.* 1999;84:677-687.

38. Rourke K, Edwards AV. Submandibular secretory and vascular responses to stimulation of the parasympathetic innervation in anethetized cats. *J Appl Physiol.* 2000;89:1964-1970.

39. Yasui T, Karita K, Izumi H, Tamai M. Correlation between vasodilatation and secretion in the lacrimal gland elicited by stimulation of the cornea and facial nerve root of the cat. *Invest Ophthalmol Vis Sci.* 1997;38:2476-2482.

40. Parod RJ, Putney JW, Jr. An alpha-adrenergic receptor mechanism controlling potassium permeability in the rat lacrimal gland acinar cell. *J Physiol.* 1978;281:359-369.

41. Parod RJ, Dambach GE, Putney JW, Jr. Membrane potential changes in lacrimal gland acinar cells elicited by carbachol and epinephrine. *J Pharmacol Exp Ther.* 1980;213:473-479.

42. Nikkinen A, Uusitalo H, Lehtosalo JI, Palkama A. Distribution of adrenergic nerves in the lacrimal glands of guinea-pig and rat. *Exp Eye Res.* 1985;40:751-756.

43. Seifert P, Stuppi S, Spitznas M, Weihe E. Differential distribution of neuronal markers and neuropeptides in the human lacrimal gland. *Graefe's Arch Clin Exp Ophthalmol.* 1996;234:232-240.

44. Matsumoto Y, Tanabe T, Ueda S. Kawata M. Immunohistochemical and enzymehistochemical studies of peptidergic, aminergic and cholinergic innervation of the lacrimal gland of the monkey (Macaca fuscata). *J Auton Nerv Syst.* 1992;37:207-214.

45. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Eng J Med.* 1993;329:2002-2012.

46. Marletta MA. Nitric oxide synthase: aspects concerning structure and catalysis. *Cell.* 1994;23;927-930.

47. Nathan C, Xie Q. Nitric oxide synthase: roles, tolls, and controls. *Cell.* 1994;78:915-918.

48. Schmidt HH, Walter U. NO at work. *Cell.* 1994;78:919-925.

49. Ayajiki K, Okamura T, Toda N. Nitric oxide mediates, and acetylcholine modulates, neurally induced relaxation of bovine cerebral arteries. *Neuroscience.* 1993;54:819-825.

50. Akaike T, Yoshida M, Miyamoto Y, et al. Antagonistic action of imidazolineoxyl *N*-oxides against endothelium-derived relaxing factor/NO through a radical reaction. *Biochemistry.* 1993;32:827-832.

51. Buxton IL, Cheek DJ, Eckman D, Westfall DP, Sanders KM, Keef KD. N^G-nitro-L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ Res.* 1993;72:387-395.

52. Cartwright JE, Whitley GS, Johnstone AP. Endothelial cell adhesion molecule expression and lymphocyte adhesion to endothelial cells: effect of nitric oxide. *Exp Cell Res.* 1997;235:431-434.

53. Dun NJ, Dun SL, Wu SY, Forstermann U. Nitric oxide synthase immunoreactivity in rat superior cervical ganglia and adrenal glands. *Neurosci Lett.* 1993;158:51-54.

54. Goadsby PJ, Uddman R, Edvinsson L. Cerebral vasodilatation in the cat involves nitric oxide from parasympathetic nerves. *Brain Res.* 1996;707:110-118.

55. Gonzalez C, Estrada C. Nitric oxide mediates the neurogenic vasodilation of bovine cerebral arteries. *J Cereb Blood Flow Metab.* 1991;11:366-370.

56. Miles K, Quintaus J, Chelmicka-Schorr E, Arnason BGW. The sympathetic nervous system modulates antibody response to thymus-independent antigens. *J Neuroimmunol.* 1981;1:101-105.

57. Kosonen O, Kankaanranta H, Vuorinen P, Moilanen E. Inhibition of human lymphocyte proliferation by nitric oxide-releasing oxatriazole derivatives. *Eur J Pharmacol.* 1997;337:55-61.

58. Liew FY. Regulation of lymphocyte functions by nitric oxide [Review]. *Curr Opin Immunol.* 1995;7:396-399.

59. Lumme A, Vanhatalo S, Soinila S. Axonal transport of nitric oxide synthase in autonomic nerves. *J Auton Nerv Syst.* 1996;56:207-214.

60. Runer T, Cervin A, Lindberg S, Uddman R. Nitric oxide is a regulator of mucociliary activity in the upper respiratory tract. *Otolaryngol Head Neck Surg.* 1998;119:278-287.

61. Stamler JS. Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell.* 1994;78:931-936.

62. Takai N, Uchihashi K, Higuchi K, Yoshida Y, Yamaguchi M. Localization of neuronal-constitutive nitric oxide synthase and secretory regulation by nitric oxide in the rat submandibular and sublingual glands. *Arch Oral Bio.* 1999;44:745-750.

63. Soinila S, Vanhatalo S, Lumme A, Back N, Soinila J. Nitric oxide synthase in the autonomic and sensory ganglia innervating the submandibular salivary gland. *Microsc Res Tech.* 1996;35:32-43.

64. Alm P, Ekstrom J, Larsson B, Tobin G, Andersson KE. Nitric oxide synthase immunoreactive nerves in rat and ferret salivary glands, and effects of denervation. *Histochem J.* 1997;29:669-676.

65. Lohinai Z, Szekely AD, Soos L, Feher E. Distribution of nitric oxide synthase containing elements in the feline submandibular gland. *Neurosci Lett.* 1995;192:9-12.

66. Konttinen YT, Platts LAM, Tuominen S, et al. Role of nitric oxide in Sjogren's syndrom. *Arthritis Rheum.* 1997;40:875-883.

67. Demer JL, Poukens V, Miller JM, Micevych P. Innervation of extraocular pulley smooth muscle in monkeys and humans. *Invest Ophthalmol Vis Sci.* 1997;38:1774-1785.

68. Nozaki K, Moskowitz MA, Maynard KI, et al. Possible origins and distribution of immunoreactive nitric oxide synthase-containing nerve fibers in cerebral arteries. *J Cereb Blood Flow Metab.* 1993;13:70-79.

69. Zhu BS, Gibbins IL, Blessing WW. Preganglionic parasympathetic neurons projecting to the sphenopalatine ganglion contain nitric oxide synthase in the rabbit. *Brain Res.* 1997;769:168-172.

70. Lee SH, Iwanaga T, Hoshi O, et al. Nitric oxide synthase in rat nasal mucosa; immunohistochemical and histochemical localization. *Acta Otolaryngol.* 1995;115:823-829.

71. Hanazawa T, Tanaka K, Chiba T, et al. Distribution and origin of nitric oxide synthase-containing nerve fibers in human nasal mucosa. *Acta Otolaryngol.* 1997;117:735-737.

72. Buckle AD, Parker SJ, Bloom SR, Edwards, AV. The role of nitric oxide in the control of protein secretion in the submandibular gland of the cat. *Exp Physiol.* 1995;80:1019-1030.

73. Edwards AV, Tobin G, Ekstrom J, Bloom SR. Nitric oxide and release of the peptide VIP from parasympathetic terminals in the submandibular gland of the anaesthetized cat. *Exp Physiol.* 1996;81:349-359.

74. Tobin G, Edwards AV, Bloom SR, Ekstrom J. Nitric oxide in the control of submandibular salivary gland function in the anaesthetized ferret. *Exp Physiol.* 1997;82:825-836.

75. Edwards AV, Garrett JR. Nitric oxide-related vasodilator response to parasympathetic stimulation of the submandibular gland in the cat. *J Physiol.* 1993;464:379-392.

76. Hanna SJ, Edwards AV. The role of nitric oxide in the control of protein secretion in the parotid gland of anaesthetized sheep. *Exp Physiol.* 1998;83:533-544.

77. Lee SH, Iwanaga T, Hoshi O, Adachi I, Fujita T. Nitric oxide synthase in rat nasal mucosa; Immunohistochemical and histochemical localization. *Acta Otolaryngol.* 1995;115:823-829.

78. Ding C, Walcott B, Keyser KT. Neuronal and endothelial nitric oxide synthase are expressed in the mouse lacrimal gland (Abstract). *Invest Ophthalmol Vis Sci.* 2000;41(Suppl):S61.

79. Ding C, Walcott B, Keyser KT. Neuronal nitric oxide synthase is expressed in the mouse lacrimal gland and in cells of the pterygopalatine ganglion (Abstract). *Cornea.* 2000;19(Suppl):S83.

80. Ding C, Walcott B, Keyser KT. Neuronal nitric oxide synthase and the sympathetic innervation of the mouse lacrimal gland (Abstract). *Invest Ophthalmol Vis Sci.* 2001;42(Suppl):S259.

81. Ding C, Walcott B, Keyser KT. Neuronal nitric oxide synthase and the autonomic innervation of the mouse lacrimal gland. *Invest Ophthalmol Vis Sci.* 2001.42:2789-2795.

82. Faraci FM. Role of nitric oxide in regulation of basilar artery tone in vivo. *Am J Physiol.* 1990;259:H1216-1221.

83. Matsuda H, Kusakabe T, Kawakami T, et al. Coexistence of nitric oxide synthase and neuropeptides in the mouse vomeronasal organ demonstrated by a combination of double immunofluorescence labeling and a multiple dye filter. *Brain Res.* 1996;712:35-39.

84. Richman DP, Arnason BGW. Nicotinic acetylcholine receptor: evidence for a functional distinct receptor on human lymphocytes. *Proc Natl Acad Sci USA.* 1979;76:4632-4635.

85. Schuman EM, Madison DV. Nitric oxide and synaptic function. *Annu Rev Neurosci.* 1994;17:153-183.

86. Grider JR. Interplay of VIP and nitric oxide in regulation of the descending relaxation phases of peristalsis. *Am J Physiol.* 1993;264:G334-340.

87. Grider JR, Murthy KS, Jin JG, Makhlouf GM. Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. *Am J Physiol.* 1992;262:G774-778.

88. Ribera J, Marsal J, Casanovas A, Hukkanen M, Tarabal O, Esquerda JE. Nitric oxide synthase in rat neuromuscular junctions and in nerve terminals of *Torpedo* electric organ: its role as regulator of acetylcholine release. *J Neurosci Res.* 1998;51:90-102.

89. Shibuki K, Okada D. Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. *Nature.* 1991;349:326-328.

90. Haley JE, Wilcos GL, Chapman PF. The role of nitric oxide in hippocampal long-term potentiation. *Neuron.* 1992;8:211-216.

91. Quinson N, Miolan JP, Niel JP. Muscarinic receptor activation is a prerequisite for the endogenous release of nitric oxide modulating nicotinic transmission within the coeliac ganglion in the rabbit. *Neuroscience.* 2000;95:1129-1138.

92. Yamada K, Kushiku K, Yamada H, et al. Contribution of nitric oxide to the presynaptic inhibition by endothelin ETB receptor of the canine stellate ganglionic transmission. *J Pharmacol Exp Ther.* 1999;290:1175-1181.

93. Kukovetz WR, Holzmann S, Wurm A, Poch B. Evidence for cyclic GMP-mediated relaxant effects of nitro-compounds in coronary smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol.* 1979;310:129-138.

94. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and veins is nitric oxide. *Proc Natl Acad Sci. USA.* 1987;84:9265-9269.

95. Mollace V, Salvemini D, Anggard E, Vane J. Nitric oxide from vascular smooth muscle cells: regulation of platelet reactivity and smooth muscle cell guanylate cyclase. *Br J Pharmacol.* 1991;104:633-638.

96. Knowles RG, Palacios M, Palmer RMJ, et al. Formation of nitric oxide from oxide from L-arginine in the central nervous system, a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc Natl Acad Sci. USA.* 1989;86:5159-5162.

97. Light DB, Corbin JD, Stanton BA. Dual ion-channel regulation by cyclic GMP and cyclic GMP-dependent protein kinase. *Nature.* 1990;344:336-339.

98. Kaupp UB. The cyclic nucleotide-gated channels of vertebrate photoreceptors and olfactory epithelium. *Trends Neurosci.* 1991;14:150-157.

99. Rapoport RM, Draznin MB, Murad F. Endothelium-dependent relaxation in rat thoracic aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature.* 1983;306:174-176.

100. Sato T, Kamata Y, Irifune M, Nishikawa T. Inhibitory effect of several nitric oxide-generating compounds on purified Na^+, K^+-ATPase activity from porcine cerebral cortex. *J Neurochem.* 1997;68:1312-1318.

101. Shoker AS, Yang H, Murabit MA, Jamil H, al-Ghoul A, Okasha K. Analysis of the in vitro effect of exogenous nitric oxide on human lymphocytes. *Mol Cell Biochem.* 1997;171:75-83.

102. Shuler RL, Laskin DL, Gardner CR, Feder LS, Laskin JD. Lymphocyte-mediated nitric oxide production by rat endothelial cells. *J Leukoc Bio.* 1995;57:116-121.

103. Walcott B, Claros N, Patel A, Brink PR. Age-related decrease in innervation density of the lacrimal gland in mouse models of Sjögren's Syndrome. *Adv Exp Med Bio.* 1998;438:917-923.

104. Zoukhri D, Hodges RR, Dartt DA. Lacrimal gland innervation is not altered with the onset and progression of disease in a murine model of Sjögren's Syndrome. *Clin Immunol Immunopathol.* 1998;89:126-133.

105. Walcott B, McLean JR. Catecholamine-containing neurons and lymphoid cells in a lacrimal gland of the pigeon. *Brain Res.* 1985;328:129-137.

106. Walcott B, Sibony PA, Keyser KT. Neuropeptides and the innervation of the avian lacrimal gland. *Invest Ophthalmol Vis Sci.* 1989;30:1666-1674.

107. Dartt DA. Signal transduction and control of lacrimal gland protein secretin: a review. *Curr Eye Res.* 1989;8:619-636.

108. Brink PR, Peterson E, Banach K, Walcott B. Electrophysiological evidence for reduced water flow from lacrimal gland acinar epithelium of NZB/NFW F1 mice. *Adv Exp Med Bio.* 1998;438:209-219.

109. Mircheff AK. Lacrimal fluid and electrolyte secretion: a review. *Curr Eye Res.* 1989;8:607-617.

110. Lemp MA. Epidemiology and classification of dry eye. *Avd Exp Med Bio.* 1998;438:791-803.

111. Kessler HS. A laboratory model for Sjögren's Syndrome. *Am J Pathol.* 1968;52:671-678.

112. Kessler HS, Cubberly M, Manski W. Eye changes in autoimmune NZB and NZB×NZW mice. *Arch Ophthalmol.* 1971;85:211-219.

113. Tsubota K. Reflex tearing in dry eye not associated with Sjögren's syndrome [Review]. *Adv Exp Med Bio.* 1998;438:903-907.

APPENDIX

APPROVAL OF THE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

NOTICE OF APPROVAL

DATE: August 31, 2001

TO: Kent Keyser, Ph.D.
WORB-658 4390
FAX: 934-5725

FROM: Clinton J. Grubbs, Ph.D., Chairman
Institutional Animal Care and Use Committee

SUBJECT: Modification of Neural Control of the Lacrimal Gland in Mouse. NEI Small Grants for Pilot Research (RO3) (NIH) 010605758

On August 29, 2001, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described: additional 200 mice; no procedural changes. The following species and numbers of animals reflect this modification

Species	Use Category	Number in Category
Mice	A	200

Animal use is scheduled for review on 6/30/02. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 010605758 when ordering animals or in any correspondance with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

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