UNIVERSITY OF SZEGED

DOCTORAL SCHOOL OF CLINICAL MEDICINE

Department of Ophthalmology

PhD THESIS

Adrenergic regulation of lacrimal gland ductal fluid secretion: role and intracellular mechanisms of α-adrenergic stimulation

Dóra Szarka MSc

Supervisor:

Edit Tóth-Molnár MD PhD

Szeged, Hungary

2021

TABLE OF CONTENTS

LIST OF FULL PAPERS RELATED TO THE THESIS

Szarka D, Elekes G, Berczeli O, Vizvári E, Szalay L, Ding C, Tálosi L, Tóth-Molnár E.
Alpha-Adrenergic Agonists Stimulate Fluid Secretion in Lacrimal Gland Ducts.
Invest Ophthalmol Vis Sci 2020 Dec 1;61(14):3. doi: 10.1167/iovs.61.14.3.
Scimago: Q1/D1 Impact factor: 3.46 (2019)

Berczeli O, **Szarka D**, Elekes G, Vizvári E, Szalay L, Almássy J, Tálosi L, Ding C, Tóth-Molnár E.
The regulatory role of vasoactive intestinal peptide in lacrimal gland ductal fluid secretion: A new piece of the puzzle in tear production.
Mol Vis 2020 Dec 6;26:780-788. eCollection
Scimago: Q2 Impact factor: 2.21 (2019)

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)
BMY-7378	8-[2-[4-(methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
eNOS	Endothelial nitric oxide synthase
FURA2-AM	Fura-2 pentakis(acetoxymethyl) ester
KO	Knock out
L-NAME	N omega-Nitro-L-arginine methyl ester hydrochloride
LV	Luminal volume
MAPK	Mitogen-activated protein kinase
NKCC1	Na^+-K^+-$2Cl^-$ cotransporter 1
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
ROI	Region of interest
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
VIP	Vasoactive intestinal peptide

1. INTRODUCTION

Tear film is a complex mixture of substances secreted from multiple sources on the ocular surface, including the main and accessory lacrimal glands, the Meibomian glands, and the goblet cells [1]. The appropriate amount and composition of tear film is critical for a healthy, intact ocular surface. Tear fluid has a number of important functions. As the outermost element of the visual system, it contributes in the maintenance of high-quality vision and plays an important role in ocular surface defense from microbial infection, atmospheric and chemical irritants, as well as temperature changes and humidity. It is not a homogenous structure and it is classically divided into three dynamic layers: lipid layer, aqueous layer and mucus layer. All these layers have several important functions [2,3].

The outermost part of the tear film, the lipid layer is secreted by the Meibomian, Zeiss and Moll glands. The lipid layer provides a smooth optical surface for the cornea, retards evaporation and prevents a spillover of the tear fluid at the lid margins [4].

The innermost layer is the mucus layer. It is secreted mostly by the conjunctival goblet cells. The mucus layer lubricates and protects the cornea, and prevents desiccation and bacterial contamination. The corneal epithelium is hydrophobic, so the hydrophilic layer, created by the mucus, let the spread of the aqueous layer over the ocular surface. The mucus layer is not tightly attached to the epithelial layer, but rather attaches to the glycocalyx and moves across the cornea. This allows the mucus to spread evenly and prevents damage to the epithelium during blinking.

The aqueous layer is located between the lipid layer and the mucus layer. There is an apparent line between the lipid and the aqueous layer while only a continuous transition can be found between the aqueous and the mucus layer. The aqueous layer contains inorganic salts, glucose, proteins and antibodies for corneal wound repair and for the defense against infection and it protects cells from the damage caused by free radicals as well. Furthermore, it plays a vital nutritive role in the transport of substances necessary for corneal metabolism and regeneration. The cornea is avascular for transparency, therefore it requires a nonvascular way for the supply of oxygen, electrolytes, growth factors, and nutrients and for the removal of metabolic products such as carbon dioxide from the ocular surface [2, 5]. Predominant amount of the aqueous layer is produced by the lacrimal gland.

The lacrimal gland is a tubuloacinar exocrine gland what secretes electrolytes, water, proteins, and mucins - known as lacrimal gland fluid - into the tear film [6]. In humans and rabbits, it is located within the bony orbit of the eye. In rats and mice, the main lacrimal gland is exorbital, lying just below the ear. The lacrimal gland is composed of three major cell types: acinar, ductal, and myoepithelial cells [7]. The myoepithelial cells surround the basal side of both acinar and duct cells. These cells are flat stellate cells that form a network beneath the nerves and blood vessels and interfacing between the acinar and duct cells and the extracellular space. The myoepithelial cells do not have fluid secretory function. The main secretory cell is the acinar cell that comprises about 80% of the gland. Acinar cells are pyramidal shaped cells that are linked together by tight junctions. The tight junctions separate the apical from the basolateral plasma membranes and are responsible for the polarization of the acinar cell. The basolateral membrane contains the receptors for neurotransmitters, neuropeptides, and growth factors that are involved in the secretory function. It also contains ion transport proteins and ion channels for initiating electrolyte and water secretion. The apical membrane also contains different ion transport proteins and ion channels and the secretory granules that deliver proteins into the lumen. Lumens of the acinar cells converge to form the interlobular and intralobular excretory ducts [6, 7].

The ductal cells are also linked by tight junctions resulting in polarization of the cells and contributes to one-way secretion of lacrimal fluids. Most research have been focusing on the function of acinar epithelial cells and much less efforts have been paid to the research of the ductal system even though its important role in lacrimal gland function has been assumed for a long time [7, 8, 9]. Lack of functional experimental methods hindered the research of physiology and pharmacology of lacrimal gland ducts. An isolated duct model was developed in our laboratory with the modified adaptation of the method used in pancreas duct research [10, 11]. Using this model and a video-microscopy technique, experimental evidence of fluid secretion of rabbit lacrimal gland ducts was given, confirming the important role of ducts in tear secretion [12]. The isolated duct segment model is also suitable for investigation of intracellular mechanisms of the duct system. Using this model our research group identified and studied the functional activity of several ion transporters in the polarized duct cells. Fluorescence measurements of intracellular calcium concentration confirmed the presence of Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger on the basolateral side of rabbit lacrimal gland ducts [10]. Functional role of NKCC1 was investigated with ammonium-pulse technique in rabbit lacrimal gland ducts [13]. CFTR KO mice model were used to study the role of

parasympathetic regulation in lacrimal gland duct function and to study the role of CFTR in the secretory mechanism of ducts [14]. Carbachol- and VIP-induced fluid secretory experiments provided direct functional evidence of the importance of parasympathetic regulation in lacrimal gland ducts [15].

Regardless of the results mentioned above, autonomic regulation of the ductal function is far not fully explored. Parasympathetic pathways are rated as the main regulatory system of lacrimal gland function while sympathetic effects have been supposed to play an indirect role through blood flow regulation [16-19]. There are increasing evidence, however, that sympathetic stimulation - apart from the hemodynamic effects - plays a direct and important role in the protein secretion of the lacrimal gland [20, 21]. Although earlier reports suggested that both α and β adrenergic agonists could result in protein secretory response in whole lacrimal gland pieces of mouse, the role of α-adrenergic receptors is suggested to be more relevant [22]. Furthermore, intracellular mechanisms mediating α-adrenergic stimulation in lacrimal gland involve additional pathways beside the conventional route through activation of phospholipase C [23]. This conception is supported by the well documented fact, that the dominant α-adrenergic receptor subtype present in the lacrimal gland is the α_{1D} and not the most common α_{1A} or α_{1B} subtypes [24, 25].

The intracellular mechanisms of α_{1D} adrenergic receptor activation are not clearly understood. Additionally, involvement of the NO/cGMP pathway was suggested in the phenylephrine-induced protein secretion of lacrimal gland in rat [24]. However, all these results were obtained from studies investigating the effect of adrenergic stimulation on acinar cells or on whole lacrimal gland pieces. Therefore, the effect of adrenergic stimulation as well as the intracellular mechanisms underlying this process in ducts of lacrimal glands are completely unknown.

2. AIMS OF THE STUDY

Although there is a growing amount of information about the function of adrenergic innervation in lacrimal gland, the available studies provide data particularly on protein secretion of acinar cells. The role of adrenergic effect in the regulation of lacrimal gland ductal fluid secretion is unknown. Therefore, aims of the present study were:

1) to investigate the effect of adrenergic stimulation on fluid secretion of isolated lacrimal gland duct segments

2) to assess the type and subtype of the involved adrenergic receptors

3) to study the underlying intracellular mechanisms.

3. MATERIALS AND METHODS

3.1 Animals

FVB/N mice were used in our study. The animals were 12-16 weeks old and weigh of 18-22 g. Both gender were used in a 1:1 ratio for all experiments. Mice were narcotized intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg) and euthanized with pentobarbital overdose (100 mg/kg) then the exorbital lacrimal glands were dissected. All experiments were conducted in compliance with the statement of the Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmic and Vision Research. The protocol has been approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary and conformed to the Directive 2010/63/EU of the European Parliament.

3.2 Solutions and chemicals

Media and its supplements for lacrimal gland duct isolation and culture (Dulbecco's modified Eagle medium, McCoy's 5A tissue culture medium, fetal calf serum, glutamine and bovine serum albumin), poly-l-lysine, phenylephrine, isoprenaline, propranolol, phentolamine, noradrenaline, carbachol (carbamoylcholine chloride), eNOS inhibitor L-NAME, guanylyl cyclase inhibitor ODQ, intracellular Ca^{2+} chelator BAPTA-AM and α_{1D}-adrenergic receptor inhibitor BMY-7378 were purchased from Sigma-Aldrich Corp. (Budapest, Hungary). Collagenase was purchased from Worthington Biochemical Corp. (Lakewood, NJ, USA). FURA2-AM was purchased from Invitrogen (Waltham, MA, USA). The compositions of solutions used in our experiments are summarized in **Table 1**. The standard HCO_3^- /CO_2^- buffered solution was gassed with 5% CO_2/95% O_2 at 37 °C.

Compound	Content of Solutions			
	HCO_3^- /CO_2^- buffered solution	**Isolation solution**	**Storage solution**	**Culture solution**
NaCl (mM)	115			
KCl (mM)	5			
$MgCl_2$ (mM)	1			
$CaCl_2$ (mM)	1			
D-Glucose (mM)	10			
$NaHCO_3$ (mM)	25			
Dulbecco's Modified Eagle Medium		X	X	
Collagenase (U/ml)		100		
Bovine serum albumin (mg/ml)		1	0.03	
McCoy's 5A Tissue Culture Medium				X
Fetal calf serum (vol/vol %)				10
Glutamine (mM)				2

Table 1. Composition of the solutions used in our experiments

3.3 Isolation and culture of lacrimal duct segments

Lacrimal glands were dissected and transferred to a sterile 1.5 ml Eppendorf tube containing a cold (+4°C) storage solution. Isolation solution was prepared and injected into the interstitium of the glands. Lacrimal gland tissues were transferred to a Petri dish containing 3 ml of isolation solution for incubation in 37 °C incubator gassed with 5% CO_2 for 7 minutes. Enzymatically digested tissue samples were washed 3 times with storage solution. The tissue was allowed to rest for 30 minutes at 4 °C. Lacrimal gland tissue samples were transferred to a glass microscope slide using a Pasteur pipette and viewed under stereo microscope. Intralobular and interlobular ducts were microdissected with 26-gauge × 0.5 inch medical stainless steel needles. Isolated duct segments were kept in short term culture for 8-10 hours in a 37 °C incubator gassed with 5% CO_2.

3.4 Measurement of fluid secretion of lacrimal gland ducts

Ends of isolated, viable duct segments seal during the applied 8-10 hours long culture (**Figure 1)**. Sealed duct segments form a closed intraluminal space. Secretory processes of duct epithelium into the closed intraluminal space result in swelling of the ducts as the luminal space fills with the secreted fluid (**Figure 2**). The change in duct volume can be analyzed using the video microscopic method. The technique was originally described for the investigation of pancreatic ducts and was adapted by our laboratory for the measurement of ductal fluid secretion [12, 26]. Cultured lacrimal gland duct segments were carefully transferred to a coverslip pretreated with diluted poly-l-lysine (diluted in distilled water, ratio = 1:9). The coverslip formed the base of a perfusion chamber mounted on an inverted microscope (Olympus Ltd., Budapest, Hungary). The chamber was perfused with solutions via an infusion pump at approximately 2.5 ml/minute at 37°C. Ducts were visualized at high magnification (200 x objective). Bright-field images were acquired at set time intervals (1image/minute) using a charge-coupled digital camera device coupled to a personal computer. Both the duration of experiments and the time intervals between images were defined in Xcellence (Olympus Ltd.) imaging software. An image series in tagged image file format was generated containing all of the images collected from the same experiment. Scion Image (Scion Corporation, Frederick, MD, USA) software was used to obtain values and analyze changes in the area corresponding to the luminal space in each image. The initial lumen length (L_0) and the initial lumen area (A_0) were measured directly from the pixel intensities on the first image. The lumen diameter was calculated assuming the cylindrical setup of the duct, from the formula $2R = A_0/L_0$. The luminal surface area was calculated as $2\pi R_0 L_0$, also assuming cylindrical geometry. Measurements from subsequent individual images were normalized to the first lumen area in the series (A_0) thus giving values for the relative area ($A_R = A/A_0$). Relative luminal volume ($V_R = V/V_0$) of the ducts was then calculated from the relative image area. These calculations were done using Scion Image and Microsoft Excel software (Microsoft, Redmond, WA, USA)

The investigated compounds were added to the perfusate after 10 minutes superfusion with HCO_3^- / CO_2 buffered solution. At the end of each experiment, perfusion was changed to hypotonic solution for 5 minutes in order to confirm epithelial integrity. Complete sealing was proved by rapid swelling of the ducts as a response to hypotonic challenge. Data obtained from ducts not showing swelling response to hypotonic solution were discarded.

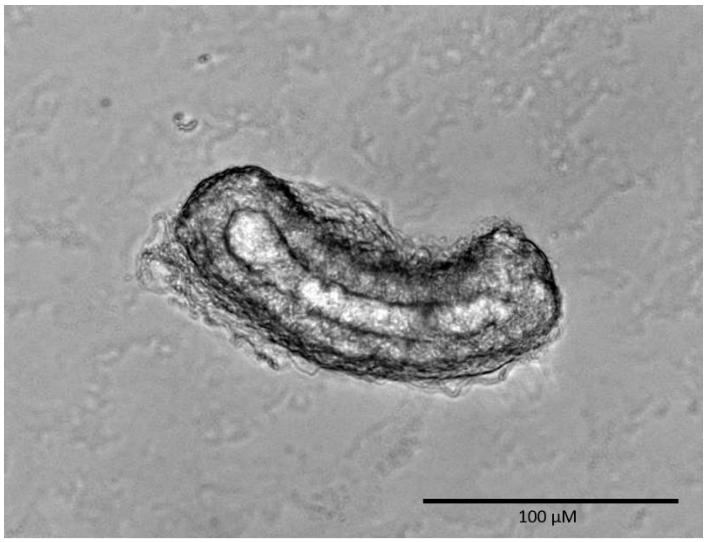

Figure 1. Bright filed imaging of a duct segment isolated from mouse lacrimal gland. Ends of the isolated duct seal during inculation, forming a closed intraluminal space.

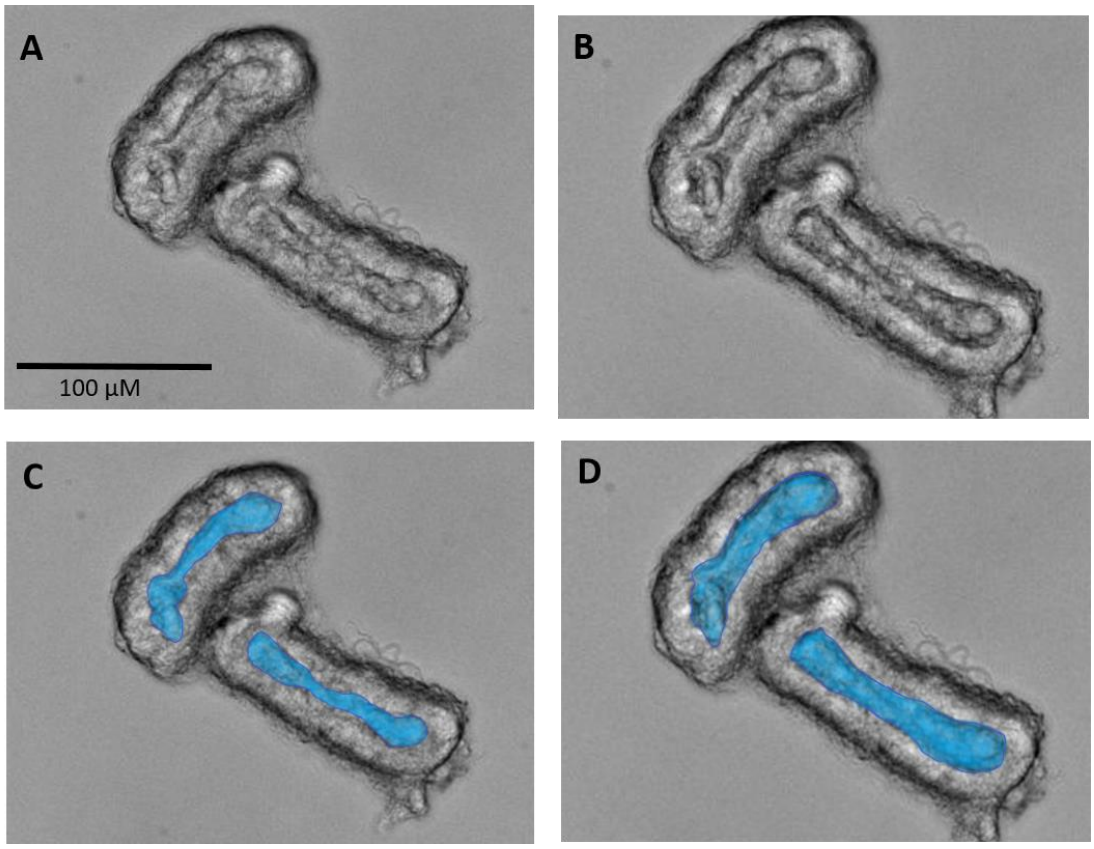

Figure 2. Swelling response of isolated mouse lacrimal gland duct segments. A: unstimulated ducts; B: the same ducts following 10 minutes of phenylephrine stimulation. Intraluminal spaces are marked with blue in C and D panels. Apart from blue color- marked intraluminal spaces, C and D panels are identical with A and B panels, respectively.

3.5 Measurement of intracellular Ca^{2+} level with microfluorophotometry

Cultured lacrimal gland ducts were attached to a coverslip as described in the previous section. The ducts were bathed in HCO_3^- / CO_2 buffered solution at 37°C and loaded with the Ca^{2+}-sensitive fluorescent dye FURA-2AM (5 μM) for 30 minutes in 37°C incubator gassed with 5 % CO_2. After loading, the ducts were continuously perfused with solutions at a rate of 4 to 5 ml/minute. Changes in intracellular Ca^{2+} level were measured using an imaging system (Xcellence; Olympus). Five small ROIs of 5 to 10 cells were selected in each intact duct. ROIs were excited with light at wavelengths of 340 nm and 380 nm, and the 380/340 fluorescence emission ratio was measured at 510 nm. One intracellular Ca^{2+} measurement was obtained per second.

3.6 Statistical analysis

For the analysis of ductal fluid secretion, effects of the stimulatory agents (phenylephrine, isoproterenol, noradrenaline) were taken into account as “fixed effects”. The effect of the individual “duct" and the “duct and effects of phenylephrine / isoproterenol / noradrenaline interaction” (we assumed that the value of the effect of the stimulatory compounds depend on the individual duct) were taken into account as random effects in the model. For the investigation of the inhibitory effect of L-NAME, ODQ and BMY-7378, data were expressed as the percent change of the LV above baseline LV (baseline LV was considered 1.0). A mixed ANOVA model was used for statistics, by using SigmaPlot version 12.5 (Systat Software Inc., San Jose, CA, USA), results were presented as means ± SEM. A ‘p’ value of less, than 0.05 was regarded as significant.

4. RESULTS

4.1 Effect of adrenergic agonists on fluid secretion of lacrimal gland ducts

4.1.1 Noradrenaline

Isolated mouse lacrimal gland ducts were used for the investigation of the effect of various adrenergic agonist on ductal fluid secretion. In the first series of experiments, ducts were stimulated with various concentrations (5, 10 or 20 μM) of the natural adrenergic agonist noradrenaline to determine the secretory response and dose-response relationship. Noradrenaline stimulates both α- and β-adrenergic receptors causing a complete adrenergic upset. Application of noradrenaline initiated a dose-dependent, rapid fluid secretory response (**Table 2**). The most effective concentration of noradrenaline proved to be 10 μM, higher concentration (20 μM) did not result in further increase in the secretory response of the investigated ducts.

Concentration of noradrenaline	**Initiated fluid secretion in the first 10 minutes of stimulation**
5 μM	120.7 ± 19.1 pl/minute/mm^2
10 μM	189.6 ± 13.9 pl/minute/mm^2
20 μM	181.5 ± 11.7 pl/minute/mm^2

Table 2. Effect of different doses of noradrenaline on secretory response of isolated lacrimal gland ducts. Data were obtained from six ducts isolated from at least three different animals in each series and are presented as means ± SEM.

4.1.2 Phenylephrine

To analyze the role of various adrenergic receptors in the observed adrenergic secretory response, effects of selective α_1 and β_1 adrenergic stimulations were investigated. In the α_1-adrenergic studies, ducts were stimulated with phenylephrine. Various concentrations (5, 10 or 20 μM) were used to determine the secretory response and dose-response relationship. To

ensure the blockade of β-adrenergic receptors, phenylephrine was administered in the presence of β-adrenergic antagonist propranolol (1 μM). Secretory response of ducts given to phenylephrine was similar to those caused by noradrenaline: application of phenylephrine evoked a rapid fluid secretory reaction (**Table 3**). The optimal concentration of phenylephrine found to be 10 μM: higher concentration (20 μM) did not result in any further increase in the fluid secretion of the ducts. Therefore, this concentration (10 μM) was used throughout the additional phenylephrine experiments. It is important to mention, that no statistically significant difference was detected between the extent of the fluid secretory rates evoked by phenylephrine in the presence of propranolol vs. noradrenaline ($p=0.42$) and the kinetics of these stimulated secretions were also similar.

Concentration of phenylephrine	**Initiated fluid secretion in the first 10 minutes of stimulation**
5 μM	116.5 ± 19.1 pl/minute/mm^2
10 μM	187.8 ± 26.8 pl/minute/mm^2
20 μM	182.1 ± 22.5 pl/minute/mm^2

Table 3. Effect of different doses of phenylephrine on secretory response of isolated lacrimal gland ducts. Data were obtained from six ducts isolated from at least three different animals in each series and are presented as means ± SEM.

Earlier studies demonstrated, that α-adrenergic receptor subtype present in the acinar epithelial cells of lacrimal gland is the α_{1D} [24, 25]. Therefore, we investigated whether this receptor subtype is involved in the α-adrenergic secretory response of mouse lacrimal gland ducts. To answer that question, the effect of α_{1D}-adrenergic blockade on phenylephrine evoked ductal fluid secretion was investigated in the next series of experiments. Duct segments were pre-incubated with different doses of selective α_{1D} receptor antagonist BMY-7378 (1, 10, 100 and 200 μM) for 30 minutes and then phenylephrine (10 μM) was added to the superfusate containing α_{1D} receptor antagonist.

BMY-7378 reduced phenylephrine-induced ductal fluid secretion in a dose-dependent manner (**Table 4**).

Concentration of BMY-7378	Phenylephrine (10μM) evoked fluid secretion above baseline LV
1 μM (n=5)	58.27 ± 7.12 %
10 μM (n=5)	42.24 ± 6.51 %
100 μM (n=7)	7.64 ± 9.68 %
200 μM (n=5)	7.69 ± 8.71 %

Table 4. Effect of different doses of BMY-7378 on phenylephrine evoked secretory response of isolated lacrimal gland ducts (baseline LV means unstimulated state). Number of ducts isolated from 3 different animals is indicated as "n". Data are presented as means ± SEM.

The difference between baseline LV and the LV measured following phenylephrine stimulation in the presence of 100 μM BMY-7378 was statistically not significant (p=0.081). Therefore, administration of 100 μM BMY-7378 completely abolished phenylephrine-induced ductal fluid secretion proving the role of α_{1D} -adrenergic receptors in the observed secretory response (**Figure 3**).

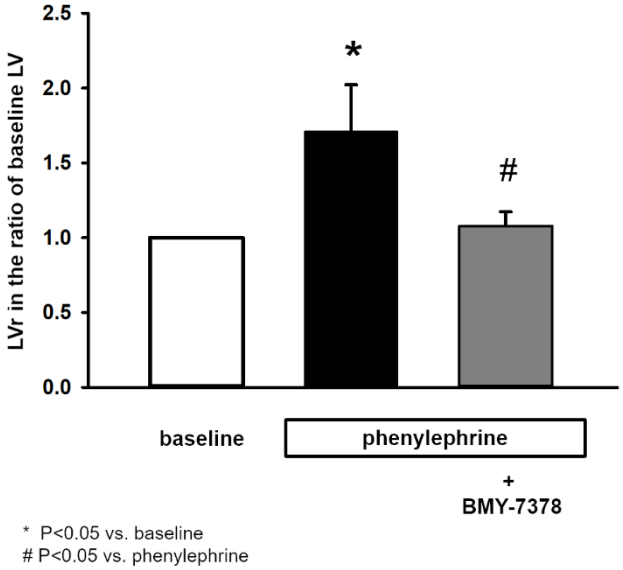

Figure 3. Effect of BMY-7378 pretreatment on phenylephrine induced secretory response of isolated lacrimal gland ducts. Isolated ducts were stimulated with phenylephrine (10 μM) either in the presence or in the absence of α_{1D}-receptor antagonist BMY-7378 (100 μM). Secretory response of ducts was measured with videomicroscopy. Changes in relative luminal volume (LVr) are shown. Data were obtained at least from six ducts isolated from three different animals in each series and are presented as means ± SEM.

4.1.3 Isoproterenol

Effect of β-adrenergic stimulation on ductal fluid secretion was also investigated. β-adrenergic agonist isoproterenol was administered in the presence of α-adrenergic antagonist phentolamine (10 μM) to ensure the selective β-adrenergic stimulation. Isoproterenol failed to elicit any detectable secretory effect in all applied concentrations (50, 100 or 200 μM; n=5, in each series of experiments). Secretory rate was -0.8±19.7 pl/minute/mm^2 in the first 10 minutes of stimulation in case of the highest isoproterenol concentration (200 μM).

4.2 Investigation of intracellular mechanisms underlying phenylephrine-induced ductal fluid secretion

4.2.1 Effect of eNOS inhibitor L-NAME on phenylephrine-induced ductal fluid secretion

Since the mechanisms underlying α_{1D}-adrenergic receptor stimulation involve NO/cGMP pathway [24] in acinar cells, the role of this intracellular pathway was investigated in the next series of experiments. Lacrimal gland ducts were pre-incubated with different doses of eNOS inhibitor L-NAME (1, 10, 100 or 200μM) for 30 minutes and then 10 μM of phenylephrine was added to the bath containing eNOS inhibitor.

Phenylephrine-evoked ductal fluid secretion was reduced by L-NAME in a dose-dependent manner (**Table 5**).

Concentration of L-NAME	**Phenylephrine (10 μM) evoked fluid secretion above baseline LV**
1 μM (n=5)	53.01 ± 8.20 %
10 μM (n=5)	33.50 ± 10.02 %
100 μM (n=8)	21.82 ± 13.52 %
200 μM (n=6)	22.14 ± 14.1 %

Table 5. Effect of different doses of L-NAME on phenylephrine evoked secretory response of isolated lacrimal gland ducts (baseline LV means unstimulated state). Number of ducts isolated from 3 different animals is indicated as "n". Data are presented as means ± SEM.

However, even at the maximal inhibitory effect of L-NAME, a significant difference ($p=0.023$) was found between baseline LV and LV measured following phenylephrine stimulation in the presence of L-NAME (**Figure 4**). These results suggest that although administration of L-NAME reduced, but not completely abolished the phenylephrine-induced fluid secretion of isolated lacrimal gland ducts.

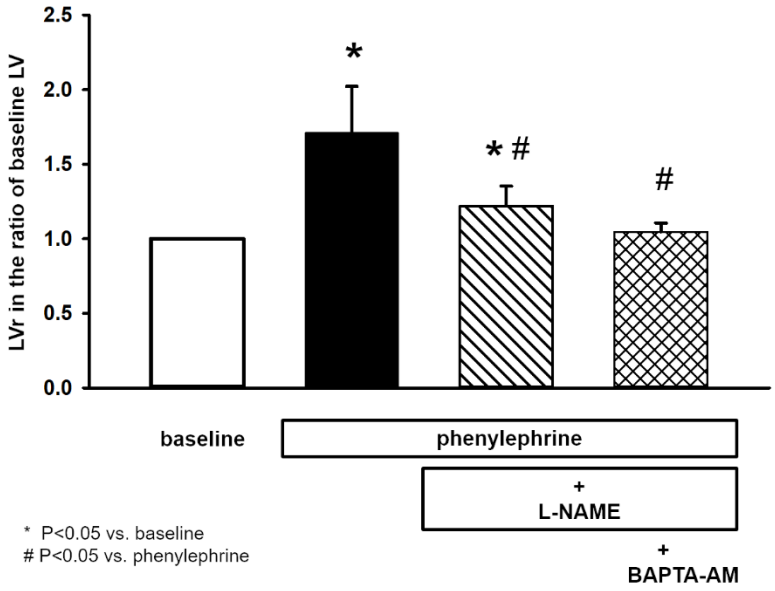

Figure 4. Effect of L-NAME and L-NAME/BAPTA-AM pretreatment on phenylephrine induced secretory response of isolated lacrimal gland ducts. Isolated ducts were stimulated with phenylephrine (10 μM) either in the absence of eNOS inhibitor L-NAME or in the presence of L-NAME (100 μM) alone or combined with Ca^{2+} chelator BAPTA-AM (10 μM). Secretory response of ducts was measured with video-microscopy. Changes in relative luminal volume (LVr) are shown. Data were obtained at least from six ducts isolated from three different animals in each series and are presented as means ± SEM.

4.2.2 Effect of guanylyl cyclase inhibitor ODQ on phenylephrine-induced ductal fluid secretion

To investigate the role of cGMP pathway in phenylephrine-induced ductal fluid secretion, lacrimal gland ducts were pre-incubated with different doses of guanylyl cyclase inhibitor ODQ (0.1, 1, 10 or 100 μM) for 30 minutes and then 10 μM of phenylephrine was added to the bath containing guanylyl cyclase inhibitor.

Inhibition of guanylyl cyclase with ODQ decreased phenylephrine-induced LV increase in a dose dependent manner (**Table 6**).

Concentration of ODQ	Phenylephrine (10 μM) evoked fluid secretion above baseline LV
0.1 μM (n=5)	70.90 ± 9.07 %
1 μM (n=5)	55.28 ± 10.01 %
10 μM (n=7)	21.78 ± 2.97 %
100 μM (n=7)	23.13 ± 5.20 %

Table 6. Effect of different doses of ODQ on phenylephrine evoked secretory response of isolated lacrimal gland ducts (baseline LV means unstimulated state). Number of ducts isolated from 3 different animals is indicated as "n". Data are presented as means ± SEM.

Maximal inhibition occurred at 10 μM ODQ concentration (21.78± 2.97 % above baseline LV). Although the inhibitory effect of ODQ was visible, a significant difference (p=0.0008) was proved between baseline LV and LV measured following phenylephrine stimulation in the presence of ODQ (**Figure 5**). Effect of ODQ administration was similar to the one that L-NAME produced in the previous experiments: it reduced, but not completely inhibited phenylephrine-induced ductal fluid secretion.

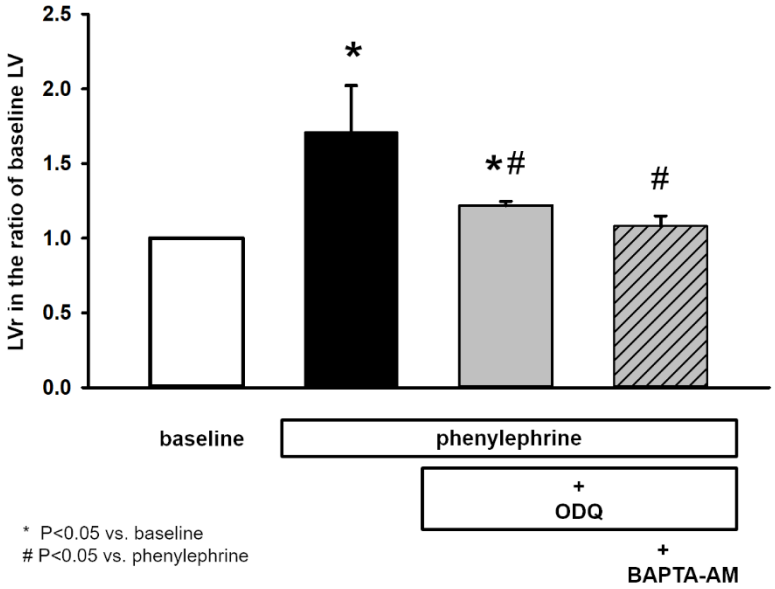

Figure 5. Effect of ODQ and ODQ/BAPTA-AM pretreatment on phenylephrine induced secretory response of isolated lacrimal gland ducts. Isolated ducts were stimulated with phenylephrine (10 μM) either in the absence of guanylyl cyclase inhibitor ODQ or in the presence of ODQ (10 μM) alone or combined with Ca^{2+} chelator BAPTA-AM (10 μM). Secretory response of ducts was measured with video-microscopy. Changes in relative luminal volume (LVr) are shown. Data were obtained at least from six ducts isolated from three different animals in each series and are presented as means ± SEM.

4.2.3 Phenylephrine-evoked Ca^{2+} signaling in isolated lacrimal gland duct segments

While α_{1D} receptor blockage with BMY-7378 completely abolished phenylephrine-induced ductal fluid secretion, inhibition of eNOS or guanylyl cyclase considerably reduced but couldn't completely block it. We hypothesized in the background of this phenomenon that the elevation of intracellular Ca^{2+} level as a consequence of α_{1D} adrenergic receptor activation may contribute to the fluid secretion of the ducts.

To investigate this theory, in the next series of experiments changes of intracellular Ca^{2+} level were measured in response to phenylephrine stimulation. In these experiments, applied concentration of phenylephrine was 10 μM similarly to the fluid secretion experiments. Stimulation of α_1-adrenergic receptors by phenylephrine resulted in a small, but statistically significant increase in intracellular Ca^{2+} level of ducts ($p=0.012$). The extent of this increase

was much smaller, compared to the response we observed previously during carbachol stimulation in epithelial cells of isolated mouse lacrimal gland ducts (**Figure 6**) [14].

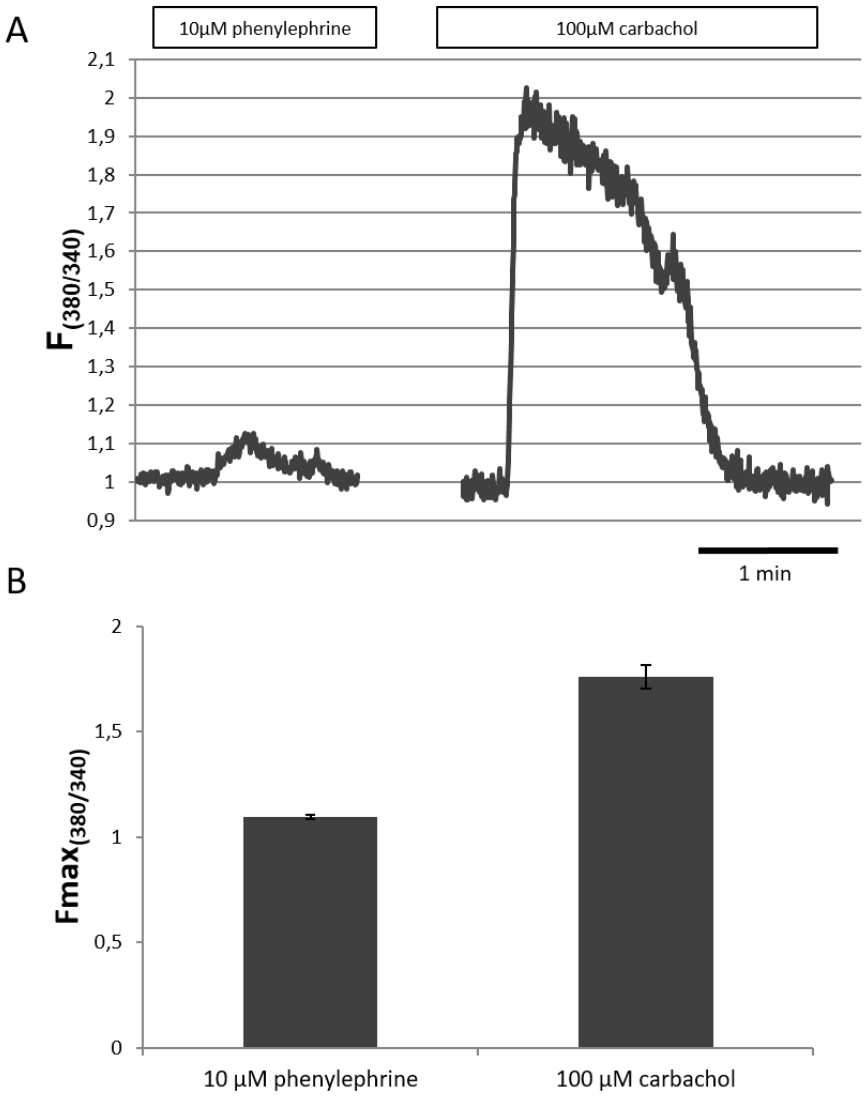

Figure 6. Effects of phenylephrine and carbachol on intracellular Ca^{2+} level in cells of isolated mouse lacrimal gland ducts. Ducts were preincubated with Ca^{2+}-sensitive fluorescent dye FURA 2AM (5 µM) and then stimulated either with phenylephrine (10 µM) or with carbachol (100 µM). (A) Representative recordings of the micro-fluorescence experiments: effect of phenylephrine (10 µM) on intracellular calcium level in lacrimal gland duct cells (left curve). Carbachol (100 µM) was used as a positive control in these experiments (right curve) (F380/340: 380/340 nm fluorescence emission ratio). (B) Maximum values of the 380/340 nm fluorescence emission ratios (Fmax(380/340): maximum value of the 380/340 nm fluorescence emission ratio).

4.2.4 Effect of co-administration of L-NAME with Ca^{2+}-chelator BAPTA-AM on phenylephrine-induced ductal fluid secretion

In contrast to BMY-7378, eNOS inhibitor L-NAME considerably reduced but not completely abolished phenylephrine-induced ductal fluid secretion. To investigate the potential role of phenylephrine-evoked elevation of intracellular Ca^{2+} level, effect of L-NAME inhibition on phenylephrine-induced secretion was investigated in the presence of intracellular Ca^{2+}-chelator BAPTA-AM.

In these experiments, isolated ducts were pre-incubated with the most effective dose of L-NAME (10 mM) and BAPTA-AM (10 μM). Co-administration of L-NAME and BAPTA-AM completely blocked phenylephrine-induced ductal fluid secretion (LV change: 2.1±4.8% above baseline LV, p=0.67) (**Figure 4**).

4.2.5 Effect of co-administration of ODQ with Ca^{2+}-chelator BAPTA-AM on phenylephrine-induced ductal fluid secretion

Based on similar considerations (ie. further investigation of reduced but not completely abolished phenylephrine-induced ductal fluid secretion in ODQ experiments) phenylephrine-induced secretion was also studied in the combined presence of ODQ and intracellular Ca^{2+}-chelator BAPTA-AM. Isolated ducts were pre-incubated with ODQ (100 μM) and BAPTA-AM (10 μM) in these experiments. A complete inhibition of phenylephrine-induced ductal fluid secretion was observed following co-administration of ODQ and BAPTA-AM: change of LV was negligible and non-significant compared to baseline value (LV change: 3.1± 2.5% above baseline LV, p=0.63) (**Figure 5**).

5. DISCUSSION

Tear secretion is regulated by the autonomic nervous system. Fibers from both the parasympathetic as well as the sympathetic ganglia innervate the gland and exert positive and negative control over the secretion. Sensory nerves are also present in lacrimal gland [16, 18]. While each individual cell may not be innervated, gap junctions chemically and electrically connect neighboring cells [27, 28]. Neurotransmitters are released from the stimulated parasympathetic nerves are acethylcholine and VIP. Sympathetic nerves contain the neurotransmitters noradrenaline and neuropeptide Y [18]. Parasympathetic pathways are rated as the main regulatory system of lacrimal gland function [16, 17]. Role of parasympathetic stimulation on ductal fluid secretion was revealed by our laboratory: secretory effects of carbachol and VIP in isolated lacrimal gland ducts were proved and analyzed in our previous studies [10, 12, 14, 15].

Beside the generally accepted decisive role of parasympathetic innervation, there are accumulating experimental evidence about the direct effect of the sympathetic system on lacrimal gland function [20-25, 29]. The presumptive role of adrenergic regulation was further confirmed in studies focusing on protein secretion of acinar cells and distribution of adrenergic receptors in lacrimal gland [22, 23, 29, 30]. However, all results, suggesting the role of sympathetic regulation were obtained from studies performed on acinar cells, on whole lacrimal gland or gland pieces.

Lack of experimental methods suitable to examine the function of lacrimal gland ducts hindered the availability of studies focusing solely on the role of the duct system. The isolated duct model developed with the modified adaptation of the method used in pancreas duct research made possible the investigation of physiology and pharmacology of lacrimal gland ducts in our laboratory [10, 12].

In my thesis the experimental evidence of the role of adrenergic effect in the regulation of lacrimal gland ductal fluid secretion is presented. Application of the natural adrenergic transmitter noradrenaline induced a rapid and robust fluid secretion in the isolated ducts. Considering the intense response observed, sympathetic stimulation may have more functional significance than previously believed. As noradrenaline stimulates both α and β adrenergic receptors we investigated the pharmacological background of the observed secretory response. Stimulation of α-adrenergic receptors with phenylephrine in the presence of β-adrenergic

blocker propranolol resulted in a pronounced ductal fluid secretion similar to those observed during application of noradrenaline. In contrast, no detectable fluid secretion was observed by the activation of β-adrenergic receptors with isoproterenol in the presence of α-adrenergic antagonist phentolamine.

These results are in agreement with a previously published study, where high density of α-adrenergic receptors and very weak presence of β-adrenergic receptors were found in lacrimal gland ducts by immunostaining [30]. Our results solidly suggest the involvement of sympathetic nervous system in the regulation of ductal fluid secretion and the decisive role of α-adrenergic receptors in the sympathetic neurotransmission of this regulatory process.

Three types of α1-adrenergic receptors, α_{1A}, α_{1B} and α_{1D}-subtypes have been identified using pharmacological, biochemical and molecular biology methods [31]. These receptors are members of the G protein-coupled receptor superfamily. α_1-adrenergic receptors are expressed in many tissue types, most notably in vascular tissues, where they typically regulate smooth muscle contraction [32]. The lacrimal gland tissue contains both α_{1B}- and α_{1D}-adrenergic receptors. The α_{1D} is the dominant between these two subtypes, while no α_{1A}-adrenergic receptors were detected by either binding or RT-PCR studies. Distribution of different α_1-adrenergic receptor subtypes in lacrimal gland ducts is not explored. The lacrimal gland contains 0.206 pmol of α_{1D}-adrenergic receptors/mg protein [25]. This is in comparison to rat tail artery, which has 0.138 pmol of α_{1D}-adrenergic receptors/mg protein, and rat aorta, which has 0.527 pmol of α_{1D}-adrenergic receptors/mg protein [33]. Despite widespread distribution, there is limited knowledge regarding the role of α_{1D}-adrenergic receptors in cellular functions. Few studies have linked activation of α_{1D}-adrenergic receptors to functions outside of the vascular tissue. α_{1D}-adrenergic receptors have been implicated in smooth muscle contraction in large arteries, including the aorta, iliac, and femoral arteries [34]. In addition, α_{1D}-adrenergic receptors have been shown to activate p42/p44 MAPK, leading to increased protein synthesis in arterial smooth muscle [35], fibroblasts [36] and lacrimal gland epithelial cells [24]. The role and function of α_{1D}-adrenergic receptors in lacrimal gland ducts is not known. In our experiments selective α_{1D} receptor blocker BMY-7378 could completely abolish the phenylephrine-induced ductal fluid secretion proving the involvement of this receptor subtype in the sympathetic innervation of lacrimal gland ducts as well as the important role of α_{1D}-adrenergic receptors in the regulation of ductal secretory function.

In exocrine tissues α_1-adrenergic agonists - in general - activate phospholipase C. Activation of phospholipase C generates the Ca^2-mobilizing second messenger inositol 1,4,5-

trisphosphate and the protein kinase C activator diacylglycerol [37]. Ca^2 with Ca^2/calmodulin-dependent protein kinases phosphorylates specific substrates, which lead to protein secretion. Diacylglycerol activates protein kinase C, which plays a direct role in stimulating secretion [38]. However, an increasing body of evidence shows that the α_{1D}-adrenergic receptor subtype presumably not connected to this mechanism. This receptor subtype may activate different signaling pathways and any or some of them may play role in the lacrimal gland:

1) α_{1D}-adrenergic agonists activate different protein kinase C isoforms. The effector enzyme that activate protein kinase C isoforms has not been yet described but this pathway is not connected to phospholipase C [39].

2) Activation of the stimulatory nitric oxide signaling pathway. In the lacrimal gland, eNOS and nNOS are both expressed. eNOS appeared to be localized with caveolae, nNOS is present in the nerves surrounding lacrimal gland epithelium [16, 27]. α_{1D}-adrenergic agonists stimulate eNOS to produce NO, leading to production of cGMP by guanylate cyclase causing the extracellular signal through the cell and stimulate protein secretion in rat lacrimal gland as previously described by Robin R. Hodges and coworkers [24].

3) α_{1D}-adrenergic agonists induce increase in intracellular Ca^{2+} level by a mechanism that is not yet determined but is not by production of inositol 1,3,5-trisphosphate.

The activation of signaling pathways listed above leads to further intracellular changes such as increased protein secretion and induction of p42/p44 MAPK [24] and activation of matrix metalloproteinases. Furthermore, the activation of MAPK involves activation of epidermal growth factor receptor [25].

To elucidate the intracellular mechanisms underlying α_{1D}-adrenergic stimulated ductal fluid secretion, the role of NO/cGMP pathway was investigated in our experiments. Both eNOS inhibitor L-NAME and guanylyl cyclase inhibitor ODQ reduced but couldn't completely block phenylephrine-evoked ductal fluid secretion. This finding partially differed from results obtained by Hodges et al in rat lacrimal gland acinar cells where application of either L-NAME or ODQ resulted in a complete blockade of phenylephrine-induced protein secretion [24]. Although our results proved the involvement of NO/cGMP pathway, an additional and obviously NO/cGMP pathway-independent mechanism was supposed in the background of the observed partial blockade. Since α-adrenergic stimulation is generally linked to Ca^{2+} signaling, the effect of phenylephrine on intracellular Ca^{2+} level was investigated. Phenylephrine stimulation resulted in a small but significant elevation of intracellular Ca^{2+} level. To specify

the role of observed Ca^{2+} signaling in the α-adrenergic stimulation-enhanced fluid secretion, a further series of experiments were performed. In these experiments, the participation of Ca^{2+} signaling was excluded by intracellular Ca^{2+} chelator BAPTA-AM. Under these circumstances, the blockade of NO/cGMP either by L-NAME or ODQ completely abolished the phenylephrine-induced ductal fluid secretion, showing the apparent role of a NO/cGMP pathway-independent Ca^{2+} signaling mechanism.

6. SUMMARY

In conclusion, our data strongly suggest the direct role of α adrenergic stimulation in lacrimal gland ductal fluid secretion. Both the lack of isoproterenol-induced fluid secretory response and the similar secretory effects of noradrenaline and phenylephrine suggest that the determining adrenergic pathway is via α-adrenergic receptors in mouse lacrimal gland ducts.

Inhibition of phenylephrine-induced ductal fluid secretion by α_{1D} adrenergic receptor antagonist or by reduction of fluid secretion by either eNOS or guanylyl cyclase inhibitors suggest that α-adrenergic agonists use the NO/cGMP pathway through α_{1D} receptor stimulation to increase fluid secretion, but involvement of a NO/cGMP pathway-independent Ca^{2+} signaling mechanism is also assumed.

Conclusions of the studies on adrenergic stimulation of isolated mouse lacrimal gland ducts presented in the thesis are:

1. **Our results prove the involvement of sympathetic nervous system in the regulation of ductal fluid secretion**
2. **α-adrenergic stimulation caused a rapid fluid secretory response in the isolated mouse lacrimal gland duct segments, therefore the α-adrenergic effect may play a significant direct role in the regulation of ductal fluid secretion**
3. **No detectable secretory effect was observed by the activation of β-adrenergic receptors**
4. **Selective α_{1D} receptor blocker BMY-7378 (100 μM) completely abolished phenylephrine-induced ductal fluid secretion proving the role of α_{1D} -adrenergic receptors in the observed secretory response**
5. **E-NOS inhibitor L-NAME (100 μM) and guanylyl cyclase inhibitor ODQ (10 μM) reduced, but not completely abolished the phenylephrine-induced fluid secretion of isolated lacrimal gland ducts showing the important but not exclusive role of a NO/cGMP pathway**
6. **Phenylephrine-evoked elevation of intracellular Ca^{2+} level has a minor but apparent role via Ca^{2+} signaling in the α-adrenergic stimulation-enhanced ductal fluid secretion**

All of these results support that sympathetic regulation plays a significantly greater role in the lacrimal gland ductal fluid secretion than previously thought.

REFERENCES

[1] TFOS DEWS II Tear Film Report. Ocul Surf 2017, Jul(3); 15: 366-403.

[2] Pflugfelder SC, Stern ME. Biological functions of tear film. Exp Eye Res 2020, 197: 108-115.

[3] Holland EJ, Mannis MJ, Lee WB. Ocular surface disease : cornea, conjunctiva and tear film. 2013, London, Elsevier/Saunders, 1st Edition, pp. 12-39.

[4] Cwiklik L. Tear film lipid layer: A molecular level view. Biochim Biophys Acta 2016, 1858(10): 2421-2430.

[5] Sullivan DA, Stern ME, Tsubota K, Dartt DA, Sullivan RM, Bromberg BB. (Eds) Lacrimal gland, tear film, and dry eye syndromes 3 : basic science and clinical relevance. Advances in experimental medicine and biology. 2002, New York: Kluwer Academic/Plenum Pub. pp. 3-16.

[6] Obata H. Anatomy and histopathology of the human lacrimal gland. Cornea 2006, Dec; 25(10 Suppl 1):S82-9.

[7] Alexander JH, van Lennep EW, Young JA. Water and electrolyte secretion by the exorbital lacrimal gland of the rat studied by micropuncture and catheterization techniques. Pflugers Arch 1972, 337(4): 299-309.

[8] Dartt DA, Moller M, and Poulsen JH. Lacrimal gland electrolyte and water secretion in the rabbit: localization and role of (Na^+/K^+)-activated ATPase. J Physiol 1981, 321: 57-69.

[9] Mircheff AK. Control of lacrimal gland function: Water and electrolyte secretion and fluid modification. In: Albert DM, Jakobiec FA, eds. Principles and Practice in Ophthalmology. Philadelphia: WB Saunders; 1994, 466-472.

[10] Tóth-Molnár E, Venglovecz V, Ozsvári B, Rakonczay Z Jr, Varró A, Papp JG et al. New experimental method to study acid/base transporters and their regulation in lacrimal gland ductal epithelia. Invest Ophthalmol Vis Sci 2007, 48(8): 3746-55.

[11] Argent BE, Arkle S, Cullen MJ, Green R. Morphological, biochemical and secretory studies on rat pancreatic ducts maintained in tissue culture. Q J Exp Physiol 1986, 71:633–648.

[12] Katona M, Vizvári E, Németh L, Facskó A, Venglovecz V, Rakonczay Z Jr et al. Experimental evidence of fluid secretion of rabbit lacrimal gland duct epithelium. Invest Ophthalmol Vis Sci 2014, 55(7): 4360-7.

[13] Vizvári E, Katona M, Orvos P, Berczeli O, Rárosi F, Facskó A et al. Characterization of Na^+-K^+-$2Cl^-$ cotransporter activity in rabbit lacrimal gland duct cells. Invest Ophthalmol Vis Sci 2016, 57(8): 3828-35.

[14] Berczeli O, Vizvári E, Katona M, Török D, Szalay L, Rárosi F et al. Novel insight into the role of CFTR in lacrimal gland duct function in mice. Invest Ophthalmol Vis Sci 2018, 59(1): 54-62.

[15] Berczeli O, Szarka D, Elekes G, Vizvári E, Szalay L, Almássy J, et al. The regulatory role of vasoactive intestinal peptide in lacrimal gland ductal fluid secretion: A new piece of the puzzle in tear production. Mol Vis 2020, Dec 6; 26:780-788.

[16] Hodges RR, Dartt DA. Regulatory pathways in lacrimal gland epithelium. Int Rev Cytol 2003, 231: 129-96.

[17] Bromberg BB. Autonomic control of lacrimal protein secretion. Invest Ophthalmol Vis Sci 1981, 20(1): 110-6.

[18] Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. Prog Retin Eye Res 2009, 28(3): 155-77.

[19] Goldstein AM, De Palau A, Botelho SY. Inhibition and facilitation of pilocarpine-induced lacrimal flow by norepinephrine. Invest Ophthalmol 1967, 6(5): 498-511.

[20] Botelho SY, Martinez EV, Pholpramool C, Prooyen HC, Jansen JT, De Palau A. Modification of stimulated lacrimal gland flow by sympathetic nerve impulses in rabbit. Am J Physiol 1976, 230(1): 80-4.

[21] Parod RJ, Putney Jr JW. An alpha-adrenergic receptor mechanism controlling potassium permeability in the rat lacrimal gland acinar cell. J Physiol 1978, 281: 359-69.

[22] Ding C, Walcott B, Keyser KT. Sympathetic neural control of the mouse lacrimal gland. Invest Ophthalmol Vis Sci 2003, 44(4): 1513-20.

[23] Hodges RR, Dicker DM, Rose PA, Dartt DA. α_1-adrenergic and cholinergic agonists use separate signal transduction pathways in lacrimal gland. Am J Physiol 1992, 262(6 Pt 1):G1087-96.

[24] Hodges RR, Shatos MA, Tarko RS, Vrouvlianis J, Gu J, Dartt DA. Nitric oxide and cGMP mediate alpha$_{1D}$-adrenergic receptor-stimulated protein secretion and p42/p44 MAPK activation in rat lacrimal gland. Invest Ophthalmol Vis Sci 2005, 46(8): 2781-9.

[25] Chen LL, Hogdes RR, Funaki C, Zoukhri D, Gaivin RJ, Perez DM et al. The effect of α_{1D} adrenergic receptors on shedding of biologically active EGF in freshly isolated lacrimal gland epithelial cells. Am J Physiol Cell Physiol 2006, 291(5): C946–C956.

[26] Fernández-Salazar MP, Pascua P, Calvo JJ, López MA, Case RM, Stewart MC et al. Basolateral anion transport mechanisms underlying fluid secretion by mouse, rat and guinea-pig pancreatic ducts. J Physiol 2004, 556: 415–428.

[27] Michon L, Nlend Nlend R, Bavamian S, Bischoff L, Boucard N, Caille D et al. Involvement of gap junction communication in secretion. Biochim Biophys Acta 2005, 1719: 82-101.

[28] Walcott B, Moore LC, Birzgalis A, Claros N, Valiunas V, Ott T et al. Role of gap junctions in fluid secretion of lacrimal glands. Am J Physiol Cell Physiol 2002, 282(3): C501-7.

[29] Dartt DA, Rose PA, Dicker DM, Ronco LV, Hodges RR. Alpha$_1$-adrenergic agonist-stimulated protein secretion in rat exorbital lacrimal gland acini. Exp Eye Res 1994, 58(4): 423-9.

[30] Ding C, Walcott B, K.T. Keyser KT. The alpha$_1$- and beta$_1$-adrenergic modulation of lacrimal gland function in the mouse. Invest Ophthalmol Vis Sci 2007, 48(4): 1504-10.

[31] Piascik MT, Perez DM. α_1-adrenergic receptors: new insights and directions. J Pharmacol Exp Ther 2001, 298: 403–410.

[32] Hague C, Chen Z, Uberti M, Minneman KP. α_1-adrenergic receptor subtypes: non-identical triplets with different dancing partners? Life Sci 2003, 74: 411–418.

[33] Tanaka T, Zhang L, Suzuki F, Muramatsu I. Alpha-1 adrenoceptors: evaluation of receptor subtype-binding kinetics in intact arterial tissues and comparison with membrane binding. Br J Pharmacol 2004, 141:468–476.

[34] Gericke A, Martinka P, Nazarenko I, Persson PB, Patzak A. Impact of alpha 1-adrenoceptor expression on contractile properties of vascular smooth muscle cells. Am J Physiol Regul Integr Comp Physiol 2007, 293: R1215-21.

[35] Xin X, Yang N, Eckhart AD, Faber JE. Alpha 1$_D$-adrenergic receptors and mitogen-activated protein kinase mediate increased protein synthesis by arterial smooth muscle. Mol Pharmacol 1997, 51: 764–775.

[36] Waldrop BA, Mastalerz D, Piascik MT, Post GR. Alpha 1$_B$- and alpha 1$_D$-adrenergic receptors exhibit different requirements for agonist and mitogen-activated protein kinase activation to regulate growth responses in rat fibroblasts. J Pharmacol Exp Ther 2002, 300: 83–90.

[37] Mauduit P, Jammes H, Rossignol B. M3 muscarinic acetylcholine receptor coupling to PLC in rat exorbital lacrimal acinar cells. Am J Physiol Cell Physiol 1993, 264: C1550–C1560.

[38] Zoukhri D, Hodges RR, Sergheraert C, Toker A, and Dartt DA. Lacrimal gland PKC

isoforms are differentially involved in agonist-induced protein secretion. Am J Physiol Cell Physiol 1997, 272: C263–C269.

[39] Ikeda-Kurosawa C, Higashio H, Nakano M, Okuba M, Satoh YI, Kurosaka D et al. α1-Adrenoceptors relate Ca^{2+} modulation and protein secretions in rat lacrimal gland. Biochem Res 2015, 36:357-369.

ACKNOWLEDGEMENTS

First and foremost I would like to express my gratitude to my mentor and guide **Dr. Edit Tóth-Molnár** who has been a constant source of ideas, knowledge, support and encouragement. With her passion about her work and her moral values, she taught me not just how to be a better professional but also a better human being.

I am grateful to **Prof. Dr. András Varró**, the former head of Department of Pharmacology and Pharmacotherapy who gave me opportunity to work at his DepartmentI also would like to say thank you for **Prof. Dr. Péter Hegyi** and **Dr. Viktória Venglovecz** for the opportunity to do my research in their laboratory.

I would like to thank my colleagues from the **Lacrimal Gland Research Group**, **Gréta Elekes, Dr. Orsolya Berczeli, Dr. Eszter Vizvári** and **all members of the Pancreas Research Group** for their support and help during my work and also the for the days that put a smile on my face.

I am mentioning these names at the end of the section, yet these were the people who have encouraged me **my parents, my brother dr. Ádám Szarka, Renáta Bordás, Noémi Nikoletta Smaniotta**. Their constant support, not just emotionally, but actually taking care of my things, has kept me going on.

Alpha-Adrenergic Agonists Stimulate Fluid Secretion in Lacrimal Gland Ducts

Dóra Szarka,[1] Gréta Elekes,[1] Orsolya Berczeli,[1] Eszter Vizvári,[1] László Szalay,[1] Chuanqing Ding,[2] László Tálosi,[3] and Edit Tóth-Molnár[1]

[1]Department of Ophthalmology, University of Szeged, Szeged, Hungary
[2]Pharmacology & Pharmaceutical Sciences, Ophthalmology, University of Southern California, Los Angeles, California, United States
[3]Department of Pharmacognosy, University of Szeged, Szeged, Hungary

Correspondence: Edit Tóth-Molnár, Department of Ophthalmology, University of Szeged, 10-11 Korányi fasor, Szeged, H-6720, Hungary; tme@tmedit.hu.

Received: June 1, 2020
Accepted: November 1, 2020
Published: December 1, 2020

Citation: Szarka D, Elekes G, Berczeli O, et al. Alpha-adrenergic agonists stimulate fluid secretion in lacrimal gland ducts. *Invest Ophthalmol Vis Sci.* 2020;61(14):3. https://doi.org/10.1167/iovs.61.14.3

Purpose. The role of adrenergic innervation in the regulation of lacrimal gland (LG) ductal fluid secretion is unknown. The Aim of the present study was to investigate the effect of adrenergic stimulation on fluid secretion in isolated LG duct segments and to study the underlying intracellular mechanisms.

Methods. Fluid secretion of isolated mouse LG ducts was measured using video-microscopy. Effect of various adrenergic agonists (norepinephrine, phenylephrine, and isoproterenol) on fluid secretion as well as inhibitory effects of specific antagonists on adrenergic agonist-stimulated secretory response were analyzed. Changes in intracellular Ca^{2+} level [$Ca^{2+}{}_i$] were investigated with microfluorometry.

Results. Both norepinephrine and phenylephrine initiated a rapid and robust fluid secretory response, whereas isoproterenol did not cause any secretion. Phenylephrine-induced secretion was completely blocked by α_{1D}-adrenergic receptor blocker BMY-7378. The endothelial nitric oxide synthase (eNOS) inhibitor L-NAME or guanylyl cyclase inhibitor ODQ reduced but not completely abolished the phenylephrine-induced fluid secretion, whereas co-administration of Ca^{2+}-chelator BAPTA-AM resulted in a complete blockade. Phenylephrine stimulation induced a small, but statistically significant elevation in [Ca_i^{2+}].

Conclusions. Our results prove the direct role of α_1-adrenergic stimulation on LG ductal fluid secretion. Lack of isoproterenol-induced fluid secretory response suggests the absence of β-receptor mediated pathway in mouse LG ducts. Complete blockade of phenylephrine-induced fluid secretion by BMY-7378 and predominant inhibition of the secretory response either by L-NAME or ODQ suggest that α-adrenergic agonists use the NO/cGMP pathway through α_{1D} receptor. Ca^{2+} signaling independent from NO/cGMP pathway may also play an at least partial role in α-adrenergic induced ductal fluid secretion.

Keywords: lacrimal gland (LG), duct cell, adrenergic regulation, α-adrenergic

Tear film is a substantial protector of the ocular surface. A predominant amount of the aqueous layer is produced by the lacrimal gland (LG).[1] Similar to other exocrine glands, LG consists of acini and ducts.[2] Most of the research activities were focusing on the function of acinar cells and much less efforts have been paid to the investigation of the ductal system, even though an important role of the duct cells in LG function has been assumed for a long time.[3,4] Lack of experimental methods suitable to examine the function of LG ducts hindered the availability of studies focusing solely on the role of the duct system. An isolated duct model was developed in our laboratory with the modification of the method used in pancreas duct research.[5,6] Using this model and a video-microscopy technique, experimental evidence of fluid secretion of rabbit LG ducts was given, confirming the important role of ducts in tear secretion.[7] The isolated duct model is also suitable for the investigation of the regulatory mechanisms of the duct system.[5,7–9]

Autonomic regulation of the ductal function is not fully explored. Parasympathetic pathways are the main regulatory system of the LG, whereas sympathetic effects have been supposed to play indirect role through blood flow regulation.[10–12] There is increasing evidence, however, that sympathetic stimulation - apart from the hemodynamic effects - plays a direct and important role in the protein secretion of the LG.[13,14] Although earlier reports suggested that both α_1 and β_1-adrenergic agonists could result in protein secretory response in whole LG pieces of mouse and rat, the role of α_1-adrenergic receptors is expected to be more relevant.[15,16] Furthermore, intracellular mechanisms mediating α-adrenergic stimulation in LGs involve additional pathways beside the conventional route through activation of phospholipase C.[17] This conception is supported by the well documented fact that the dominant α-adrenergic receptor subtype presents in the LG is the α_{1D} and not the most common α_{1A} or α_{1B} subtypes.[18,19] Intracellular mechanisms

Table. Composition of Solution

Compound	Content of Solutions			
	HCO_3^- / CO_2^- Buffered Solution	Isolation Solution	Storage Solution	Culture Solution
NaCl, mM	115			
KCl, mM	5			
$MgCl_2$, mM	1			
$CaCl_2$, mM	1			
D-Glucose, mM	10			
$NaHCO_3$, mM	25			
Dulbecco's Modified Eagle Medium		X	X	
Collagenase, U/mL		100		
Bovine serum albumin, mg/mL		1	0.03	
McCoy's 5A Tissue Culture Medium				X
Fetal calf serum, vol/vol %				10
Glutamine, mM				2

of α_{1D}-adrenergic receptor activation are not clearly understood. Additionally, involvement of the NO/cGMP pathway was suggested in the phenylephrine-induced protein secretion of rat LG.[18] All these results were obtained from studies investigating the effect of adrenergic stimulation on acinar cells or on whole LG pieces. However, the effect of adrenergic stimulation as well as the intracellular mechanisms underlying this process in ducts of LGs are completely unknown.

Therefore, the aim of the present study was to investigate the effect of adrenergic stimulation on fluid secretion of isolated LG duct segments and to study the intracellular mechanisms underlying adrenergic stimulation.

Parts of the results in this paper have been presented in abstracts in the Annual Meetings of the Association for Research in Vision and Ophthalmology (Berczeli O., et al. IOVS 2017; 58(8): 2256; Tóth-Molnár E., et al. IOVS 2018; 59(9): 4923).

Materials and Methods

Animals

Mouse exorbital LGs dissected from 12 to 16 week old wild type FVB/N mice (a total of 56 animals) were used throughout the study. Animals were narcotized intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg), and euthanized with pentobarbital overdose (100 mg/kg).

All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary, and conformed to the Directive 2010/63/EU of the European Parliament.

Solutions and Chemicals

Media and its supplements for LG duct isolation and culture (Dulbecco's modified Eagle medium, McCoy's 5A tissue culture medium, fetal calf serum, glutamine, and bovine serum albumin), phenylephrine, isoprenaline, propranolol, phentolamine, norepinephrine, carbachol (carbamylcholine chloride), endothelial nitric oxide synthase (eNOS) inhibitor L-NAME, guanylyl cyclase inhibitor ODQ, and α_{1D}-adrenergic receptor inhibitor BMY-7378 were purchased from Sigma-Aldrich Corp. (Budapest, Hungary). Collagenase was purchased from Worthington Biochemical Corp. (Lakewood, NJ, USA). FURA2-AM was purchased from Invitrogen (Waltham, MA, USA). The compositions of solutions used in our experiments are summarized in the Table. The standard HCO_3^-/CO_2^- buffered solution was gassed with 95% O_2/5% CO_2 at 37°C.

Isolation of Ducts From Mouse LGs

Mouse LG interlobular ducts were isolated as previously described by our laboratory.[5] Briefly, LGs were dissected and transferred to a sterile small flat-bottom glass flask containing cold (4°C) storage solution. Isolation solution was injected into the interstitium of the glands and the tissue pieces were transferred to a glass flask containing 2 mL of isolation solution. Following a 15 minute incubation period in a shaking water bath at 37°C, isolation solution was removed and 5 mL of fresh cold storage (4°C) solution was added to the flask. LG tissue samples were transferred to a glass microscope slide and viewed under stereo-microscope. Following the microdissection of the ducts, the isolated duct segments were transferred to the culture solution in a Petri dish. Isolated ducts were cultured overnight in a 37°C incubator gassed with 5% CO_2.

Measurement of Ductal Fluid Secretion

Video-microscopic method was used for the measurement of ductal fluid secretion. The technique was originally described for the investigation of pancreatic ducts and was adapted by our laboratory for the measurement of ductal fluid secretion.[6,7] In brief, ends of the isolated ducts seal after 8 to 10 hours of incubation. Secretory processes of the epithelial cells result in luminal volume (LV) increase of the ducts as the closed luminal space fills with the secreted fluid. The change in ductal volume can be analyzed with videomicroscopy. Scion Image (Scion Corporation, Frederick, MD, USA) software was used to analyze and calculate changes in the LV.

Measurement of Intracellular Ca^{2+}

Ca^{2+}-sensitive fluorescent dye FURA 2AM (5 µM) was used for the measurement of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ as described earlier.[5] Changes in $[Ca^{2+}]_i$ were measured using an imaging system (Xcellence; Olympus, Budapest, Hungary). Four to 5 small areas (region of interests [ROIs]) of 5 to 10 cells in each intact duct were excited with light at 340 nm and 380 nm, and the 380 / 340

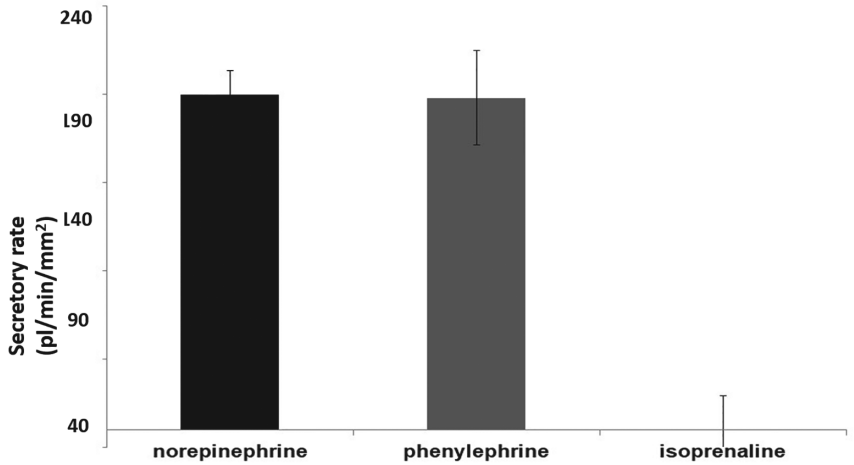

Figure 1. Effect of different adrenergic agonists on ductal fluid secretion in isolated lacrimal gland ducts. Isolated lacrimal gland ducts were stimulated with norepinephrine (10 μM), or with phenylephrine (10 μM) in the presence of propranolol (1 μM) or with isoproterenol (200 μM) in the presence of phentolamine (10 μM). Secretory response of ducts was measured with video-microscopy. Changes in relative luminal volume (Vr) are shown. Data were obtained at least from six ducts isolated from three different animals in each series and are presented as means ± SEM.

fluorescence emission ratio were measured at 510 nm. One $[Ca^{2+}]_i$ measurement was obtained per second.

Statistical Analysis

For the analysis of ductal fluid secretion, effects of the stimulatory agents (phenylephrine, isoproterenol, and norepinephrine) were considered as "fixed effects." The effect of the individual "duct" and the "duct and effects of phenylephrine/isoproterenol/ norepinephrine interaction" (we presumed the individual duct-dependent effects of the stimulatory compounds) were taken into account as random effects. For the investigation of the inhibitory effect of L-NAME, ODQ, and BMY-7378, data were expressed as the percent change of the LV above baseline LV (baseline LV was considered 1.0). A mixed ANOVA model was used for statistics, by using SigmaPlot version 12.5 (Systat Software Inc., San Jose, CA, USA), results were presented as means ± SEM. A P value of < 0.05 was regarded as significant.

Results

Effect of Adrenergic Agonists on Fluid Secretion of LG Ducts

Isolated mouse LG ducts were used for the investigation of the effect of various adrenergic agonist on ductal fluid secretion. In the first series of experiments, ducts were stimulated with various concentrations (5, 10, or 20 μM) of the natural adrenergic agonist norepinephrine (noradrenaline) to determine the secretory response and dose-response relationship. Norepinephrine stimulates both α- and β-adrenergic receptors causing a complete adrenergic upset. Application of norepinephrine initiated a dose-dependent, rapid fluid secretory response (5 μM: 120.7 ± 19.1 pl/min/mm^2; 10 μM: 189.6 ± 13.9 pl/min/mm^2; and 20 μM: 181.5 ± 11.7 pl/min/mm^2 in the first 10 minutes of stimulation). The most effective concentration of norepinephrine proved to be 10 μM (Fig. 1), higher concentration (20 μM) did not result in further increase in the secretory response of the investigated ducts. To analyze the role of various adrenergic receptors in the observed secretory response, effects of selective α_1 and β_1-adrenergic stimulations were investigated. In the α_1-adrenergic studies, ducts were stimulated with phenylephrine. Various concentrations (5, 10, or 20 μM) were used to determine the secretory response and dose-response relationship. To ensure the blockade of β-adrenergic receptors, phenylephrine was administered in the presence of β-adrenergic antagonist propranolol (1 μM). Phenylephrine stimulation caused a rapid fluid secretory response in the isolated duct segments (Fig. 1). Supplementary Video S1 demonstrates the effect of phenylephrine stimulation on ductal fluid secretion. The most effective concentration of phenylephrine found to be 10 μM (secretory rates in the first 10 minutes of stimulation: 5 μM: 116.5 ± 19.1 pl/min/mm^2; 10 μM:187.8 ± 26.8 pl/min/mm^2; and 20 μM: 182.1 ± 22.5 pl/min/mm^2). Therefore, concentration of 10 μM was used throughout the additional phenylephrine experiments. It is important to mention that no statistically significant difference was detected between the extent of the fluid secretory rates evoked by phenylephrine in the presence of propranolol versus norepinephrine ($P = 0.42$) and the kinetics of these stimulated secretions were also similar.

Effect of β-adrenergic stimulation on ductal fluid secretion was also investigated. β-adrenergic agonist isoproterenol was administered in the presence of α-adrenergic antagonist phentolamine (10 μM) to ensure the selective β-adrenergic stimulation. Isoproterenol failed to elicit any detectable secretory effect in all applied concentrations (secretory rates in the first 10 minutes of stimulation: 50 μM: −0.2 ± 11.4 pl/min/mm^2; 100 μM: 0.1 ± 17.1 pl/min/mm^2; and 200 μM: −0.8 ± 19.7 pl/min/mm^2). Figure 1 exhibits secretory result of the highest isoproterenol concentration applied (200 μM).

Effect of α_{1D}-Adrenergic Receptor Antagonist BMY-7378 on Phenylephrine-Evoked Ductal Fluid Secretion

Secretory response of isolated ducts suggested to be clearly due to the stimulation of α-adrenergic receptors in our

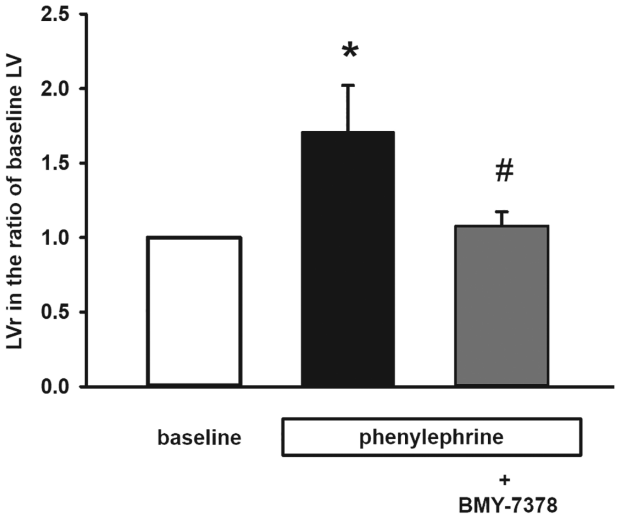

Figure 2. Effect of BMY-7378 pretreatment on phenylephrine induced secretory response of isolated lacrimal gland ducts. Isolated ducts were stimulated with phenylephrine (10 μM) either in the presence or in the absence of α_{1D}-receptor antagonist BMY-7378 (100 μM). Secretory response of ducts was measured with video-microscopy. Changes in relative luminal volume (LVr) are shown. Data were obtained at least from six ducts isolated from three different animals in each series and are presented as means ± SEM.

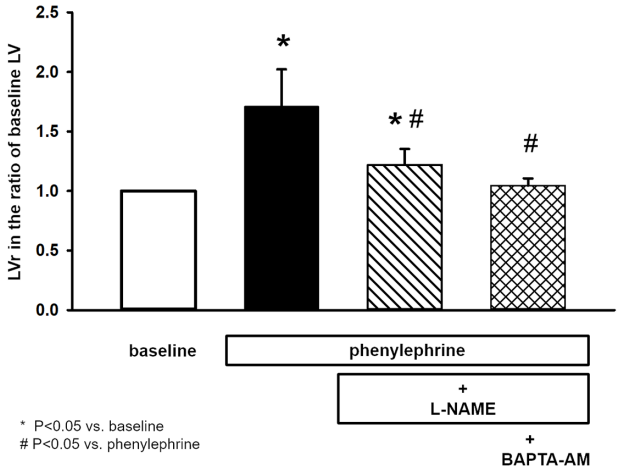

Figure 3. Effect of L-NAME and L-NAME/BAPTA-AM pretreatment on phenylephrine induced secretory response of isolated lacrimal gland ducts. Isolated ducts were stimulated with phenylephrine (10 μM) either in the absence of eNOS inhibitor L-NAME or in the presence of L-NAME (100 μM) alone or combined with Ca^{2+} chelator BAPTA-AM (10 μM). Secretory response of ducts was measured with video-microscopy. Changes in relative luminal volume (LVr) are shown. Data were obtained at least from six ducts isolated from three different animals in each series and are presented as means ± SEM.

experiments. Earlier studies demonstrated that α-adrenergic receptor subtype present in the acinar epithelial cells of LG is the α1D.[18,19] Therefore, we investigated the effect of α1D-adrenergic blockade to explore the subtype of the involved receptors in the isolated mouse LG ducts. Duct segments were pre-incubated with different doses of selective α1D receptor antagonist BMY-7378 (1, 10, 100, or 200 μM) for 30 minutes and then phenylephrine (10 μM) was added to the superfusate. BMY-7378 reduced phenylephrine-induced ductal fluid secretion in a dose-dependent manner (1 μM: 58.27 ± 7.12% above baseline LV; 10 μM: 42.24 ± 6.51% above baseline LV; 100 μM: 7.64 ± 9.68% above baseline LV; and 200 μM: 7.69 ± 8.71% above baseline LV; maximal inhibition at 100 μM [baseline LV means unstimulated state]). The difference between baseline LV and the LV measured following phenylephrine stimulation in the presence of 100 μM BMY-7378 was statistically not significant ($P = 0.081$). Therefore, administration of 100 μM BMY-7378 completely abolished phenylephrine-induced ductal fluid secretion proving the role of α_{1D}-adrenergic receptors in the observed secretory response (Fig. 2).

Effect of eNOS Inhibitor L-NAME and Guanylyl Cyclase Inhibitor ODQ on Phenylephrine-Induced Ductal Fluid Secretion

Because the mechanisms underlying α_{1D}-adrenergic receptor stimulation involve the NO/cGMP pathway, the role of this intracellular pathway was investigated in the next series of experiments. LG ducts were pre-incubated with different doses of eNOS inhibitor L-NAME (1, 10, 100, or 200 μM) for 30 minutes and then 10 μM of phenylephrine was added to the bath. Phenylephrine-evoked ductal fluid secretion was reduced by L-NAME in a dose-dependent manner (1 μM: 53.01 ± 8.2% above baseline LV; 10 μM: 33.5 ± 10.02% above baseline LV; 100 μM: 21.82 ± 13.52% above baseline LV; and 200 μM: 22.14 ± 14.10% above baseline LV; maximal inhibition at 100 μM). However, even at the maximal inhibition effect of L-NAME, a significant difference ($P = 0.023$) was found between baseline LV and LV measured following phenylephrine stimulation in the presence of L-NAME (Fig. 3). These results suggest that administration of L-NAME reduced, but not completely abolished the phenylephrine-induced fluid secretion of isolated LG ducts.

In the next series of experiments, LG ducts were pre-incubated with different doses of guanylyl cyclase inhibitor ODQ (0.1, 1, 10, or 100 μM) for 30 minutes before administration of phenylephrine (10 μM). Inhibition of guanylyl cyclase with ODQ decreased phenylephrine-induced LV increase in a dose dependent manner (0.1 μM: 70.90 ± 9.07% above baseline LV; 1 μM: 55.28 ± 10.01% above baseline LV; 10 μM: 21.78 ± 2.97% above baseline LV; and 100 μM: 23.12 ± 5.20% above baseline LV). Maximal inhibition occurred at 10 μM ODQ concentration. Although the inhibitory effect of ODQ was visible, a significant difference ($P = 0.0008$) was proved between baseline LV and LV measured following phenylephrine stimulation in the presence of ODQ (Fig. 4). Effect of ODQ administration was similar to that L-NAME produced in the previous experiments: it reduced, but not completely inhibited phenylephrine-induced ductal fluid secretion.

Phenylephrine-Evoked Ca^{2+} Signaling in Isolated LG Duct Segments

Although α_{1D} receptor blockage with BMY-7378 completely abolished phenylephrine-induced ductal fluid secretion, inhibition of eNOS or guanylyl cyclase considerably reduced but could not block it completely. We hypothesized in the background of this phenomenon that the elevation of $[Ca^{2+}]_i$

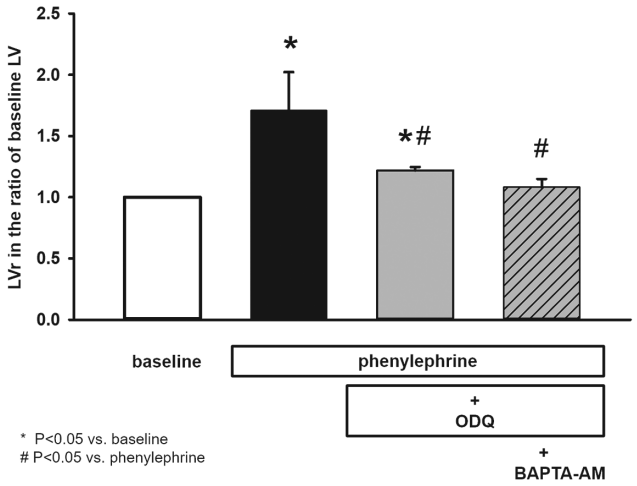

Figure 4. Effect of ODQ and ODQ/BAPTA-AM pretreatment on phenylephrine induced secretory response of isolated lacrimal gland ducts. Isolated ducts were stimulated with phenylephrine (10 µM) either in the absence of guanylyl cyclase inhibitor ODQ or in the presence of ODQ (10 µM) alone or combined with Ca^{2+} chelator BAPTA-AM (10 µM). Secretory response of ducts was measured with video-microscopy. Changes in relative luminal volume (LVr) are shown. Data were obtained at least from six ducts isolated from three different animals in each series and are presented as means ± SEM.

as a consequence of α_{1D}-adrenergic receptor activation may contribute to the fluid secretion of the ducts.

To investigate this theory, in the next series of experiments, $[Ca^{2+}]_i$ change was measured in response to phenylephrine stimulation. In these experiments, applied concentration of phenylephrine was 10 µM similarly to the fluid secretion experiments. Stimulation of α-adrenergic receptors by phenylephrine resulted in a small, but statistically significant increase in $[Ca^{2+}]_i$ ($P = 0.012$). The extent of this increase was much smaller (Fig. 5), compared to the response we observed previously during carbachol stimulation in epithelial cells of isolated mouse LG ducts.[9]

Effect of Ca^{2+} Chelator BAPTA-AM on Phenylephrine-Induced Ductal Fluid Secretion

Phenylephrine-induced ductal fluid secretion was measured in BAPTA-AM pretreated ducts in order to investigate the role of Ca^{2+} in the secretory process. Phenylephrine stimulation resulted in 169.21 ± 22.5 pl/min/mm^2 fluid secretory rate in duct cells preloaded with 10 µM of BAPTA-AM. Although this value was slightly lower compared to the secretory rate evoked by phenylephrine alone (187.8 ± 26.8 pl/min/mm^2), no statistically significant difference could be demonstrated in the fluid secretion between BAPTA-AM-treated and non-treated ducts ($P = 0.052$).

Effect of Co-Administration of L-NAME or ODQ With Ca^{2+} Chelator BAPTA-AM on Phenylephrine-Induced Ductal Fluid Secretion

In contrast to BMY-7378, eNOS inhibitor L-NAME considerably reduced but not completely abolished phenylephrine-induced ductal fluid secretion. To investigate the potential role of phenylephrine-evoked elevation of $[Ca^{2+}]_i$, the effect of L-NAME on phenylephrine-induced secretion was investigated in the presence of intracellular Ca^{2+}-chelator BAPTA-AM. In these experiments, isolated ducts were pre-incubated with the most effective dose of L-NAME (100 µM) and BAPTA-AM (10 µM). Co-administration of L-NAME and BAPTA-AM completely blocked phenylephrine-induced ductal fluid secretion (LV change: 2.1 ± 4.8% above baseline LV, $P = 0.67$).

Based on similar considerations (i.e. further investigation of reduced but not completely abolished phenylephrine-induced ductal fluid secretion in ODQ experiments) phenylephrine-induced secretion was also studied in the combined presence of ODQ and intracellular Ca^{2+}-chelator BAPTA-AM. Isolated ducts were pre-incubated with ODQ (10 µM) and BAPTA-AM (10 µM) in these experiments. A complete inhibition of phenylephrine-induced ductal fluid secretion was observed following co-administration of ODQ and BAPTA-AM: change of LV was negligible and nonsignificant compared to baseline value (LV change: 3.1 ± 2.5% above baseline LV, $P = 0.63$).

Discussion

Tear secretion is regulated by the autonomic nervous system. Besides the generally accepted decisive role of parasympathetic innervation, there is accumulating experimental evidence about the direct effect of sympathetic regulation of LG function.[13–20] The presumptive role of adrenergic regulation was confirmed in studies focusing on protein secretion of acinar cells and whole LG pieces from rat and mouse.[15,16,20,21] In the present study, role of adrenergic effect in the regulation of LG ductal fluid secretion is demonstrated. Application of the natural adrenergic transmitter norepinephrine (or noradrenaline) induced a rapid and robust fluid secretion in the isolated ducts. Considering the intense response observed, sympathetic stimulation may have more functional significance than previously believed. As norepinephrine stimulates both α and β-adrenergic receptors, the pharmacological background of the observed secretory response was investigated. Stimulation of α-adrenergic receptors with phenylephrine in the presence of β-adrenergic blocker propranolol resulted in a pronounced ductal fluid secretion similar to that observed during application of norepinephrine. In contrast, no detectable fluid secretion was observed by the activation of β-adrenergic receptors with isoproterenol in the presence of α-adrenergic antagonist phentolamine. These results are in accordance with a previously published study, where high density of α-adrenergic receptors and very weak presence of β-adrenergic receptors were found in LG ducts by immunostaining.[20] Our results strongly suggest the involvement of the sympathetic nervous system in the regulation of ductal fluid secretion. Decisive role of α-adrenergic stimulation in the sympathetic neurotransmission was demonstrated because no β-adrenergic induced fluid secretion could be observed. The α-adrenergic receptor subtype present in the acinar epithelial cells of LG is the α_{1D}, not the more common α_{1A} or α_{1B}. Selective α_{1D} receptor blocker BMY-7378 could completely abolish phenylephrine-induced ductal fluid secretion in our experiments, proving the involvement of the same receptor subtype in the sympathetic innervation of LG ducts. The intracellular mechanisms underlying α_{1D}-adrenergic receptor stimulation was found to be more complex and less clearly clarified compared to α_{1A} and α_{1B} subtypes.[22] To elucidate the intracellular mechanisms underlying α-adrenergic stimulated ductal fluid

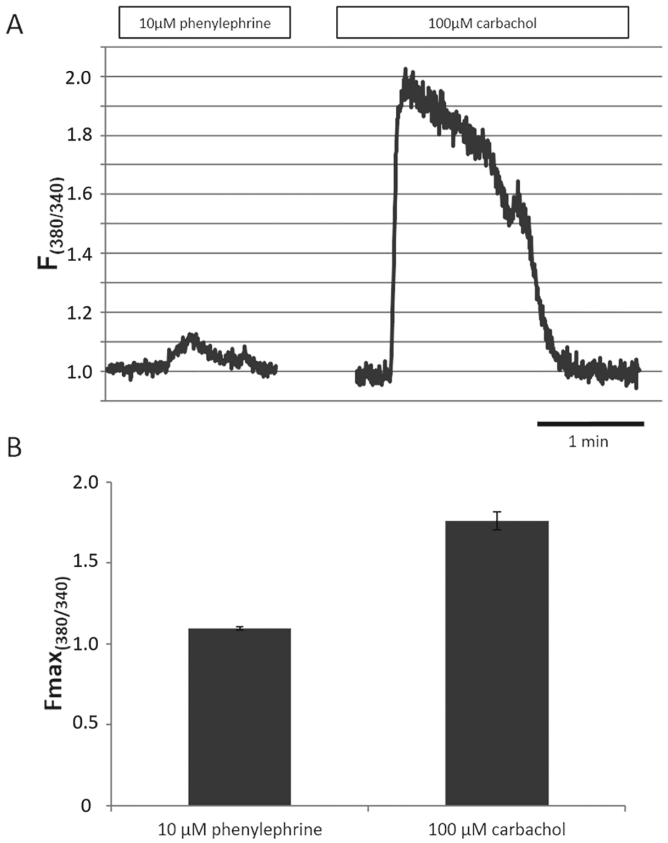

Figure 5. Effects of phenylephrine and carbachol on $[Ca^{2+}]_i$ in cells of isolated mouse lacrimal gland ducts. Ducts were preincubated with Ca^{2+}-sensitive fluorescent dye FURA 2AM (5 μM) and then stimulated either with phenylephrine (10 μM) or with carbachol (100 μM). (**A**) Representative recordings of the micro-fluorescence experiments: effect of phenylephrine (10 μM) on $[Ca^{2+}]_i$ in lacrimal gland duct cells (*left curve*). Carbachol (100 μM) was used as a positive control in these experiments (*right curve*) ($F_{380/340}$: 380/340 nm fluorescence emission ratio). (**B**) Maximum values of the 380/340 nm fluorescence emission ratios ($F_{max(380/340)}$: maximum value of the 380/340 nm fluorescence emission ratio).

secretion, the role of NO/cGMP pathway was investigated. Both eNOS inhibitor L-NAME and guanylyl cyclase inhibitor ODQ reduced but not entirely blocked phenylephrine-evoked ductal fluid secretion. These findings differed from the results obtained by Hodges et al. in rat LG acinar cells where application of either L-NAME or ODQ resulted in a complete blockade of phenylephrine-induced protein secretion.[18] An additional and obviously NO/cGMP pathway-independent mechanism was supposed in the background of the observed partial blockade. Because α-adrenergic stimulation is generally linked to Ca^{2+} signaling, the effect of phenylephrine on $[Ca^{2+}]_i$ and ductal fluid secretion was investigated.[23] Although phenylephrine stimulation resulted in a small but statistically significant elevation of $[Ca^{2+}]_i$, no statistically significant difference could be demonstrated in the fluid secretion between the Ca^{2+}-chelator BAPTA-AM-treated and nontreated ducts.

To specify the role of the observed increase in $[Ca^{2+}]_i$ in the α-adrenergic stimulation-enhanced fluid secretion, further series of experiments were performed. In these experiments, Ca^{2+} signaling was excluded by co-administration of intracellular Ca^{2+}-chelator BAPTA-AM either with L-NAME or ODQ. Under these circumstances, complete blockade of phenylephrine-induced ductal fluid secretion could be reached demonstrating the apparent role of NO/cGMP pathway-independent Ca^{2+} signaling mechanism.

Although the main intracellular event in the fluid secretion evoked by phenylephrine is the activation of the guanylyl-cyclase-cGMP pathway even though minor elevation of $[Ca^{2+}]_i$ plays some role. Blockage of the cGMP pathway alone markedly reduced but not completely abolished fluid secretion, whereas in combination with depletion of $[Ca^{2+}]_i$ resulted in complete stoppage. On the other hand, because the elevation of $[Ca^{2+}]_i$ was small, distraction of Ca^{2+} itself did not result in significant reduction in fluid secretion, although some tendency of lower secretory rates could be noticed (without reaching the statistically significant level).

In conclusion, our data strongly suggest the direct role of α-adrenergic stimulation in LG ductal fluid secretion. Lack of isoproterenol-induced fluid secretory response and the similar secretory effects of norepinephrine and phenylephrine suggest that the determining adrenergic pathway is via α_{1D}-adrenergic receptors in mouse LG ducts. Inhibition of phenylephrine-induced ductal fluid secretion by α_{1D}-adrenergic receptor antagonist or by reduction of fluid secretion by either eNOS or guanylyl cyclase inhibitors suggest that α-adrenergic agonists use the NO/cGMP pathway through α_{1D} receptor stimulation to increase fluid

secretion, but involvement of a NO/cGMP pathway-independent Ca^{2+} signaling mechanism is also assumed.

Acknowledgments

Supported by NKFIH NN 115611 (E.T.M.); EFOP-3.6.1-16-2016-00008 (ETM); SZTE ÁOK-KKA No 2018/Tóth-MolnárE (E.T.M.); NEI/NIH EY017731 (C.D.); and Webb Foundation Grant (C.D.).

Disclosure: **D. Szarka**, None; **G. Elekes**, None; **O. Berczeli**, None; **E. Vizvári**, None; **L. Szalay**, None; **C. Ding**, None; **L. Tálosi**, None; **E. Tóth-Molnár**, None

References

1. Tear Film and Ocular Surface Society International Dry Eye Workshop. FOS DEWS II Tear Film Report. *Ocul Surf*. 2017;15(3):366–403.
2. Obata H. Anatomy and histopathology of the human lacrimal gland. *Cornea*. 2006;25(10 Suppl 1):S82–S89.
3. Alexander JH, van Lennep EW, Young JA. Water and electrolyte secretion by the exorbital lacrimal gland of the rat studied by micropuncture and catheterization techniques. *Pflugers Arch*. 1972;337:299–308.
4. Dartt DA, Moller M, Poulsen JH. Lacrimal gland electrolyte and water secretion in the rabbit: localization and role of (Na+/K+)-activated ATP-ase. *J Physiol*. 1981;321:557–569.
5. Tóth-Molnár E, Venglovecz V, Ozsvari B, et al. New experimental method to study acid/base transporters and their regulation in lacrimal gland ductal epithelia. *Invest Ophthalmol Vis Sci*. 2007;48:3746–3755.
6. Fernández-Salazar MP, Pascua P, Calvo JJ, et al. Basolateral anion transport mechanisms underlying fluid secretion by mouse, rat and guinea-pig pancreatic ducts. *J Physiol*. 2004;556(2):415–428.
7. Katona M, Vizvari E, Nemeth L, et al. Experimental evidence of fluid secretion of rabbit lacrimal gland duct epithelium. *Invest Ophthalmol Vis Sci*. 2014;55:4360–4367.
8. Vizvári E, Katona M, Orvos P, et al. Characterization of Na+-K+-2Cl- cotransporter activity in rabbit lacrimal gland duct cells. *Invest Ophthalmol Vis Sci*. 2016;57:3828–3835.
9. Berczeli O, Vizvári E, Katona M, et al. Novel insight into the role of CFTR in lacrimal gland duct function in mice. *Invest Ophthalmol Vis Sci*. 2018;59(1):54–62.
10. Bromberg BB. Autonomic control of lacrimal protein secretion. *Invest Ophthalmol Vis Sci*. 1981;20:110–116.
11. 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.
12. Goldstein AM, De Palau A, Botelho SY. Inhibition and facilitation of pilocarpine-induced lacrimal flow by norepinephrine. *Invest Ophthalmol*. 1967;6:498–511.
13. Parod RJ, Putney JW. An alpha-adrenergic receptor mechanism controlling potassium permeability in the rat lacrimal gland acinar cell. *J Physiol*. 1978;281:359–369.
14. Hodges RR, Dartt DA. Regulatory pathways in lacrimal gland epithelium. *Int Rev Cytol*. 2003;231:129–196. Review.
15. Ding C, Walcott B, Keyser KT. Sympathetic neural control of the mouse lacrimal gland. *Invest Ophthalmol Vis Sci*. 2003;44(4):1513–1520.
16. Mauduit P, Herman G, Rossignol B. Protein secretion in lacrimal gland: alpha1-beta adrenergic synergism. *Am J Physiol*. 1986;250(5 Pt 1):C704–C712.
17. Hodges RR, Dicker DM, Rose PA, Dartt DA. α_1-adrenergic and cholinergic agonists use separate signal transduction pathways in lacrimal gland. *Am J Physiol*. 1992;262(6 Pt 1):G1087–G1096.
18. Hodges RR, Shatos MA, Tarko RS, Vrouvlianis J, Gu J, Dartt DA. Nitric oxide and cGMP mediate α_{1D}-adrenergic receptor-stimulated protein secretion and p42/p44 MAPK activation in rat lacrimal gland. *Invest Ophthalmol Vis Sci*. 2005;46(8):2781–2789.
19. Chen LL, Hogdes RR, Funaki C, et al. The effect of α_{1D} adrenergic receptors on shedding of biologically active EGF in freshly isolated lacrimal gland epithelial cells. *Am J Physiol Cell Physiol*. 2006;291(5):C946–C956.
20. Ding C, Walcott B, Keyser KT. Alpha 1- and beta 1- adrenergic modulation of lacrimal gland function in the mouse. *Invest Ophthalmol Vis Sci*. 2007;48:1504–1510.
21. Dartt DA, Rose PE, Dicker DM, Ronco LV, Hodges RR. α_1-adrenegid agonist-stimulated protein secretion in rat exorbital lacrimal gland acini. *Exp Eye Res*. 1994;58:423–429.
22. Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. *Prog Retin Eye Res*. 2009;28:155–177.
23. Ikeda-Kurosawa C, Higashio H, Nakano M, et al. α1-adrenoceptors relate Ca^{2+} modulation and protein secretions in rat lacrimal gland. *Biochem Res*. 2015;36:357–369.

Investigative Ophthalmology & Visual Science

Molecular Vision 2020; 26:780-788 <http://www.molvis.org/molvis/v26/780>
Received 23 May 2020 | Accepted 4 December 2020 | Published 6 December 2020

The regulatory role of vasoactive intestinal peptide in lacrimal gland ductal fluid secretion: A new piece of the puzzle in tear production

Orsolya Berczeli,[1] Dóra Szarka,[1] Gréta Elekes,[1] Eszter Vizvári,[1] László Szalay,[1] János Almássy,[2] László Tálosi,[3] Chuanqing Ding,[4] Edit Tóth-Molnár[1]

[1]Department of Ophthalmology, University of Szeged, Szeged, Hungary; [2]Department of Physiology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary; [3]Department of Pharmacognosy, University of Szeged, Szeged, Hungary; [4]Department of Pharmacology & Pharmaceutical Sciences, Ophthalmology, University of Southern California, Los Angeles, CA

Purpose: Vasoactive intestinal peptide (VIP) is an important regulator of lacrimal gland (LG) function although the effect of VIP on ductal fluid secretion is unknown. Therefore, the aim of the present study was to investigate the role of VIP in the regulation of fluid secretion of isolated LG ducts and to analyze the underlying intracellular mechanisms.
Methods: LGs from wild-type (WT) and cystic fibrosis transmembrane conductance regulator (CFTR) knockout (KO) mice were used. Immunofluorescence was applied to confirm the presence of VIP receptors termed VPAC1 and VPAC2 in LG duct cells. Ductal fluid secretion evoked by VIP (100 nM) was measured in isolated ducts using videomicroscopy. Intracellular Ca^{2+} signaling underlying VIP stimulation was investigated with microfluorometry.
Results: VIP stimulation resulted in a robust and continuous fluid secretory response in isolated duct segments originated from WT mice. In contrast, CFTR KO ducts exhibited only a weak pulse-like secretion. A small but statistically significant increase was detected in the intracellular Ca^{2+} level $[Ca^{2+}]_i$ during VIP stimulation in the WT and in CFTR KO ducts. VIP-evoked changes in $[Ca^{2+}]_i$ did not differ considerably between the WT and CFTR KO ducts.
Conclusions: These results suggest the importance of VIP in the regulation of ductal fluid secretion and the determining role of the adenylyl cyclase-cAMP-CFTR route in this process.

Although acini are the determining structures of tear fluid production, a secretory role of ducts in addition to their drainage function has been suspected for a long time [1,2]. Accumulating evidence suggests that the lacrimal gland (LG) duct system may modify the compositions of the primary acinar fluid [3,4]. Our previous experimental results provided direct evidence of the active role of the LG duct system in lacrimal fluid secretion [5]. Investigation of the underlying mechanisms revealed several components, but the regulation of ductal fluid secretion is still not fully understood. Previous reports demonstrated the contribution of vasoactive intestinal peptide (VIP) released by parasympathetic nerves in acinar protein secretion, but its impact on the fluid secretion of the LG ducts remained unknown [6]. VIP has been shown as a smooth-muscle-relaxant, vasodilator peptide in the lung [7], but it is also an important regulator of tear production in humans. This was illustrated by a case report of a patient who had a VIP-secreting metastatic pancreatic adenocarcinoma. With an 80-fold elevation of serum VIP levels, this patient had tear overproduction indicating that VIP increases tear secretion in humans, most likely by stimulating LG fluid secretion [8]. There are two known receptors for VIP termed VPAC1 and VPAC2 [9-11]. Both receptors bind VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) to some degree. The VIP–receptor interaction activates the G protein $G_s\alpha$ that stimulates adenylyl cyclase, which increases the intracellular cAMP level. cAMP, in turn, activates protein kinase A that stimulates secretion by phosphorylating its target proteins. Hodges and coworkers suggested that most, but not all, of VIP-stimulated protein secretion is cAMP dependent. Using a myristoylated protein kinase A peptide inhibitor (PKI) based on the pseudosubstrate of protein kinase A, VIP-stimulated protein secretion was shown to be inhibited by about 70% [6].

Several hormones and neurotransmitters (including VIP) with the potential to elevate cytosolic cAMP level can activate the cystic fibrosis transmembrane conductance regulator (CFTR) channel. Our previous findings strongly suggested the role of CFTR in ductal fluid secretion [12]. CFTR has been shown to play a critical role in exocrine glands, such as the pancreas, salivary, sweat glands, and airways epithelium [13-15]. Furthermore, there are several data about altered tear secretion seen in patients with cystic fibrosis implying the potential influence of CFTR [16,17]. Accumulating evidence from gene expression studies performed on rat and rabbit LGs

Correspondence to: Edit Tóth-Molnár Department of Ophthalmology University of Szeged 10-11 Korányi fasor Szeged, H-6720, Hungary; Phone: +36308864894; email: tme@tmedit.hu

Table 1. Composition of solutions.

Content of Solutions									
Compound	**HCO_3^- /CO_2^- buffered solution**		**Isolation solution**		**Storage solution**		**Culture solution**		
NaCl (mM)	115								
KCl (mM)	5								
$MgCl_2$ (mM)	1								
$CaCl_2$ (mM)	1								
D-Glucose (mM)	10								
$NaHCO_3$ (mM)	25								
Dulbecco's Modified Eagle Medium			X		X				
Collagenase (U/ml)				100					
BSA (mg/ml)				1		0.03			
McCoy's 5A Tissue Culture Medium							X		
Fetal calf serum, (vol/vol %)									10
Glutamine (mM)									2

demonstrated the predominant expression of CFTR in LG duct cells [3,18]. The main role of CFTR is regulated anion conductance in the apical membrane of many different epithelial cell types. Regulation of channel activity is predominantly via cAMP/protein kinase A signaling [19,20]. The aim of the study was to determine the effect of VIP on ductal fluid secretion and to reveal the underlying mechanisms of VIP stimulation. Parts of the results in this manuscript have been presented in abstracts in the Annual Meeting of the Association for Research in Vision and Ophthalmology [21].

METHODS

Animals: CFTR knockout (KO) mice were used throughout the experiments [22,23]. The mice were congenic on the FVB/N background. Wild-type (WT) refers to the +/+ littermates of the CFTR KO mice. The mice used in this study were 14–16 weeks old and weighed 18–22 g. The gender ratio was 1:1 for all groups. Animals were narcotized intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg) and euthanized with pentobarbital overdose (100 mg/kg).

All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged (Szeged, Hungary) and conformed to the Directive 2010/63/EU of the European Parliament and to the Hungarian Government Decree 40/2013 (II:14.). Breeding of gene-modified mice was approved under the permission No. XXII./2116/2018.

Solutions and chemicals: The compositions of solutions (for isolation, storage, and culture of isolated duct segments) used in the present studies are summarized in Table 1. Media supplements (Dulbecco's modified eagle medium [DMEM], McCoy, fetal calf serum [FCS], glutamine, and bovine serum albumin [BSA]), VIP, carbachol (carbamoylcholine chloride) and 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA-AM) were purchased from Sigma-Aldrich (Budapest, Hungary). The standard HCO_3^-/CO_2 buffered solution was gassed with 95% O_2/5% CO_2 at 37 °C. FURA2 AM was purchased from Invitrogen (Waltham, MA).

Immunofluorescence: Immunofluorescence was used to confirm the presence of VPAC1 and VPAC2 receptors in the LG. A detailed description of the method was given previously by our laboratory [12]. Fifteen µm thick cryostat sections were rehydrated by washing in tris-buffered saline (TBS, 20 mM Tris-HCl pH:7.5, 150 mM NaCl) for 5 min, then fixed in 2% paraformaldehyde (PFA). After washing the sections in TBS three times for 5 min each, the samples were permeabilized with 0,1% Triton-X in TBS for 10-15 min. The sections were blocked with 5% FCS in TBS for 1 h at room temperature followed by an overnight incubation with primary antibodies for for VPAC1 (1:1000) or VPAC2 (1:250, Abcam, Cambridge, UK) at 4 °C. The next day the samples were incubated with secondary antibody, Alexa-488 conjugated goat anti-rabbit (1:1000, Abcam) for 1 hour and Hoechst (1:1000, Sigma-Aldrich) for 20 min at room temperature. The samples were examined under a Zeiss LSM 880 confocal laser scanning microscope (Oberkochen, Germany). Images were analyzed with ImageJ (NIH, Bethesda, MD).

Isolation and culture of lacrimal duct segments: Mouse LG interlobular ducts were isolated as previously described by our laboratory [4]. In brief, LG was dissected and transferred to a sterile flat-bottom glass flask containing storage solution (4 ºC). Isolation solution was injected into the LG tissue and the pieces were transferred to a glass flask containing 2 ml of isolation solution for incubation in a shaking water bath at 37 ºC Isolation solution was removed after incubating for 25 min and 5 ml of fresh storage solution (4 ºC) was added to the flask. LG tissues were then transferred onto a glass microscope slide, and interlobular ducts were micro-dissected under a stereomicroscope and then transferred to the culture solution in a Petri dish. Isolated ducts were cultured overnight in a 37 °C incubator gassed with 5% CO_2.

Measurement of ductal fluid secretion: The videomicroscopic technique was used for the measurement of duct fluid secretion. The method was originally developed for the measurement of pancreatic duct fluid secretion and was modified by our laboratory for the investigation of LG duct secretion [5,24].

Measurement of intracellular Ca^{2+} concentration: Intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was measured using Ca^{2+}-sensitive fluorescent dye FURA2 AM (4–5 µM) as described [4]. Changes in $[Ca^{2+}]_i$ were measured using an imaging system (Xcellence; Olympus, Budapest, Hungary). Four to five small areas (regions of interests [ROIs]) of five to ten cells were excited in each intact duct with light at 340 and 380 nm, and the 380/340 fluorescence emission ratio was measured at 510 nm. Results are expressed as the maximum value of the 380/340 fluorescence emission ratio $[F_{max\ (380/340)}]$. One $[Ca^{2+}]_i$ measurement was obtained per second.

Statistical analysis: A mixed analysis of variance (ANOVA) model was applied for the calculation of ductal fluid secretion. Effects of the stimulatory compound (VIP) were taken into account as fixed effects. The effect of the individual duct and the duct and effects of VIP interaction (we assumed that the value of the effect of the stimulatory compounds depends on the individual duct) were taken into account as random effects in the model. Statistical software (SigmaPlot Systat; Software, Inc., London, England) was used to analyze the data, which were presented as means ± standard error of the mean (SEM). A p value of less than 0.05 was regarded as statistically significant.

RESULTS

Immunofluorescence: The presence of VPAC1 was dominant in the duct cells as demonstrated in Figure 1. The receptor was localized on the basolateral surface of the duct cells. Immunofluorescence staining of VPAC1 receptors revealed a mosaic pattern among different duct segments in the expression of the receptor proteins. The intensity of the fluorescence varied widely in the investigated duct segments from intense immunofluorescence to a lack of staining. No difference was detected in the immunoreactivity between the LG tissues from the WT and CFTR KO mice.

Intense VPAC2 staining was detected not only in the duct cells but also in the basolateral surface of the acinar cells (Figure 2). The intensity of the immunofluorescence staining was similar in the case of the WT and CFTR KO LGs.

VIP-induced fluid secretion in isolated LG ducts: The effect of VIP (100 nM) on ductal fluid secretion was investigated in WT and CFTR KO mouse LG ducts. A remarkable proportion of isolated ducts failed to react to the applied agent. These findings are in accordance with the results in the VPAC1 immunofluorescence studies in which the expression of the receptor protein showed a mosaic pattern, and the fluctuation of the presence of VPAC1 predicted a widely altering response of different duct segments to VIP stimulation. Obviously, all data displayed in this section were obtained from the reacting (i.e., responding with a swelling reaction) ducts.

The concentration of VIP was 100 nM in these experiments. This value was determined based on data from the literature and on our preliminary experiments in which higher concentrations of VIP (200 and 500 nM) did not result in greater effects compared to the 100 nM concentration (results are not shown). The VIP stimulation (100 nM) resulted in a robust and continuous fluid secretory response in isolated LG duct segments derived from WT mice (the secretory rate in the first 10 min of stimulation was 213.1±37.30 pl/min/mm^2, n = 7; Figure 3A). In contrast, the CFTR KO ducts exhibited only a weak pulse-like secretion in the first 5 min of stimulation (54.5±18.4 pl/min/mm^2, n = 6), followed by a plateau (Figure 3B). The VIP-induced luminal volume changes in the WT and CFTR KO ducts observed during the videomicroscopic experiments are shown in Figure 4.

VIP-induced Ca^{2+} signaling of isolated LG ducts: The CFTR KO ducts exhibited only a weak pulse-like secretion during VIP stimulation. We hypothesized in the background of this phenomenon that in addition to lacking the cAMP-CFTR pathway VIP stimulation may induce changes in $[Ca^{2+}]_l$, and this latter intracellular mechanism may also contribute to the fluid secretion of ducts. Therefore, in the next series of experiments possible effects of VIP stimulation on $[Ca^{2+}]_i$ were measured.

Intracellular Ca^{2+} homeostasis underlying VIP stimulation was measured in WT and CFTR KO duct segments. VIP stimulation (100 nM) resulted in a small but statistically

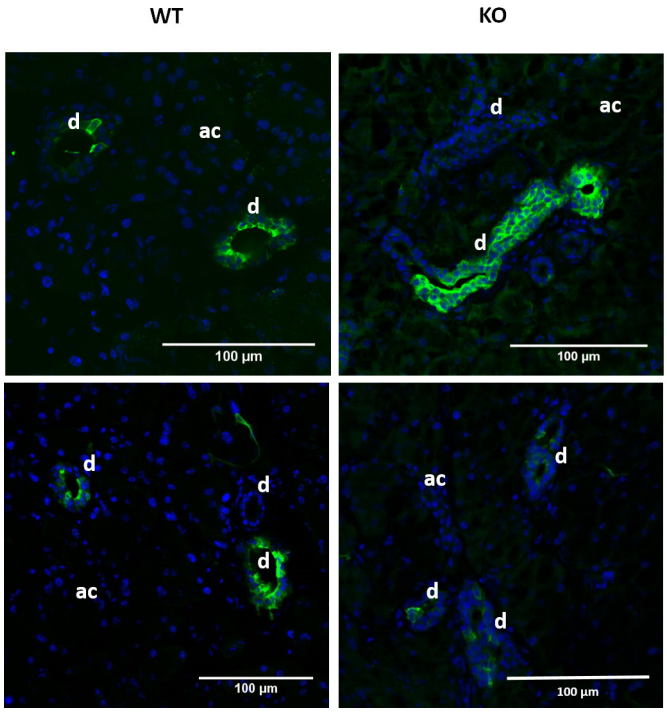

Figure 1. Immunofluorescence staining of VPAC1 receptors in lacrimal gland tissues of WT and CFTR KO mice. VPAC1 staining was more intense in ducts (d) than in acinar (ac) cells. A mosaic pattern was observed in the expression of the receptor proteins in different ducts. The intensity of the fluorescence varied widely in the investigated duct segments from intense immunofluorescence to a lack of staining. There were no statistically significant differences between the wild-type (WT) and cystic fibrosis transmembrane conductance regulator (CFTR) knockout (KO) samples. Hoechst dye was used to stain nuclei blue.

significant increase in $[Ca^{2+}]_i$ in the WT (11.6±0.70%, p = 0.001, n = 6) and CFTR KO ducts (11.1±0.50%, p = 0.002, n = 6; Figure 5). Cholinergic agonist carbachol (100 µM) was used as positive control in these experiments. The selection of the 100 µM concentration for carbachol was based on our previous experiments where a definite dose–response relation was observed in the 1–100 µM concentration range [12].

VIP-induced fluid secretion in the presence of intracellular Ca^{2+}-chelator BAPTA-AM in LG ducts isolated from CFTR KO mice: To analyze the role of Ca^{2+} signaling in VIP-induced ductal fluid secretion, the effect of VIP stimulation was investigated in the presence of intracellular Ca^{2+}-chelator BAPTA-AM. In these fluid secretion experiments, isolated ducts were preincubated with BAPTA-AM (10 µM) for 30 min before application of VIP (100 nM). A total of eight ducts

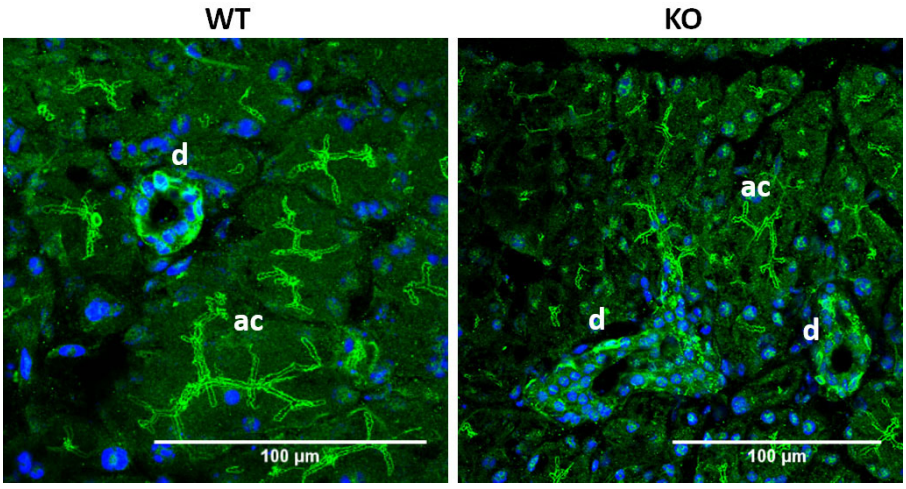

Figure 2. Immunofluorescence staining of VPAC2 receptors in lacrimal gland tissues of WT and CFTR mice. VPAC2 was observed on the basolateral surface of duct (d) and acinar (ac) cells. There were no significant differences between the wild-type (WT) and cystic fibrosis transmembrane conductance regulator (CFTR) knockout (KO) samples. Hoechst dye was used to stain nuclei blue.

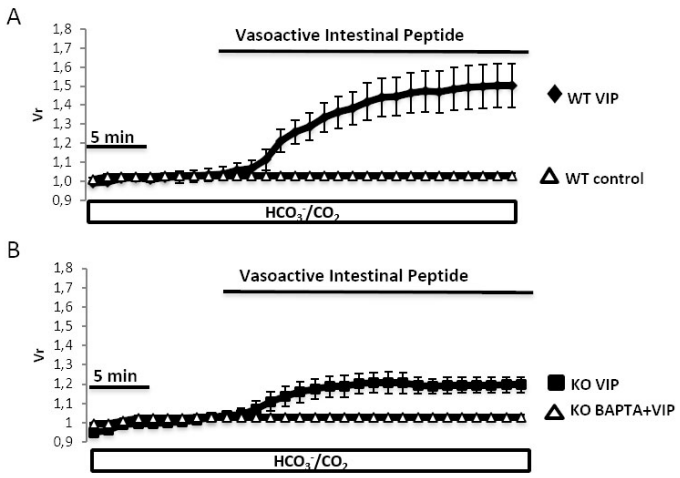

Figure 3. Effect of VIP stimulation on ductal fluid secretion in isolated lacrimal gland ducts from WT and CFTR KO mice. **A**: The wild-type (WT) ducts were exposed either to 100 nM vasoactive intestinal peptide (VIP; filled rhombus) or to no agonist (empty triangle). **B**: The cystic fibrosis transmembrane conductance regulator (CFTR) knockout (KO) ducts were exposed either to 100 nM VIP (filled square) or to 100 nM VIP following 10 µM 1,2-bis(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA-AM) pretreatment (empty triangle). Changes in relative luminal volume (Vr) are shown. Data were obtained from six to eight ducts isolated from three different animals in each series and are presented as means ± standard error of the mean (SEM).

isolated from three CFTR KO animals were investigated in these series of experiments. Following the BAPTA-AM preincubation, VIP failed to elicit an increase in secretion in any of the ducts (Figure 3B). The secretory rates were similar to the control values (i.e., no secretion was detected either in the BAPTA-AM+VIP or in the control ducts). A curve showing the secretory response of the BAPTA-AM+VIP-treated CFTR KO ducts appeared to be identical to the control curves; therefore, the results of the control experiments are not shown in Figure 3B.

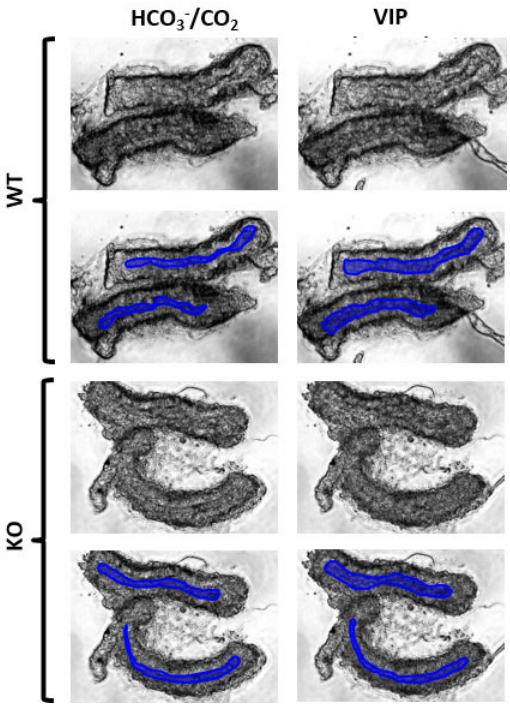

Figure 4. Representative photo series of secreting isolated lacrimal gland duct segments in response to VIP stimulation. The luminal spaces of the native images (first and third rows) are marked with blue (second and fourth rows). A strong secretory response could be shown in wild-type (WT) ducts to vasoactive intestinal peptide (VIP) stimulation as the luminal spaces were notably swollen after treatment. No remarkable changes could be detected in cystic fibrosis transmembrane conductance regulator (CFTR) knockout (KO) ducts following VIP stimulation.

DISCUSSION

In the last two decades, results of gene expression studies reaffirmed previous suggestions about the secretory role of LG duct epithelial cells, because greater expression of a wide range of transmembrane transporters could be demonstrated in the duct cells compared to acinar cells [3,25-27]. Although these results were important steps toward the clarification of duct cell function, they were unable to provide direct functional evidence. Our laboratory developed the first experimental model suitable for the investigation of lacrimal duct function which opened a new path in lacrimal duct research. Our efforts are directed toward the clarification of the role of LG ducts in the secretory process of the gland using this isolated duct model [4,5]. These viable duct segments are appropriate models not only for the investigation of the function of transmembrane transporters but also for the investigation of the regulation of secretory machinery. Although the secretory process of the duct epithelium may play an important role in tear production, our knowledge about the regulation of lacrimal duct function is limited. Tear secretion is mediated mainly by the parasympathetic system [28,29]. The anatomically and functionally predominant parasympathetic nerves can be subdivided into two different branches based on the secreted neurotransmitters. Cholinergic nerve endings release the cholinergic agonist acetylcholine, while VIP-ergic nerve terminals produce VIP. Both neurotransmitters have major effects on the secretion of the LG [28]. The available experimental evidence for the role of the parasympathetic system in LG fluid secretion is solely from intact glands or acini. We investigated the direct effect of VIP-ergic stimuli on isolated LG duct segments and explored the relationship between the CFTR chloride channel and the VIP-ergic signaling pathway.

Immunofluorescence was used to confirm the presence and localization of VPAC1 and VPAC2 proteins on LG duct cells in WT and CFTR KO mice. A previous report by Hodges et al. showed the presence of these receptors with immunostaining on the basolateral surface of acinar and duct cells in rats [6]. An apparent difference was revealed in the distribution of VPAC1 and VPAC2 receptors in mouse LG in the present study. The presence of VPAC1 was proved predominantly in ducts with immunoreactivity. In addition to the characteristically ductal localization of VPAC1 receptors, a mosaic pattern in the expression of the receptor proteins was revealed. The intensity of the immunofluorescence varied on a wide scale from intense staining to almost complete undetectability in the analyzed duct segments. VPAC2 showed a more homogeneous distribution in LG tissue: These receptors were traceable in acinar and duct cells. VPAC1 and VPAC2 were observed most prominently on the basolateral membrane of mouse LG cells. Expression patterns of VPAC1 and VPAC2 receptors differ from the presence of CFTR in mouse LG. The CFTR protein was predominant in ducts in contrast to the VPAC2 receptors, while CFTR expression

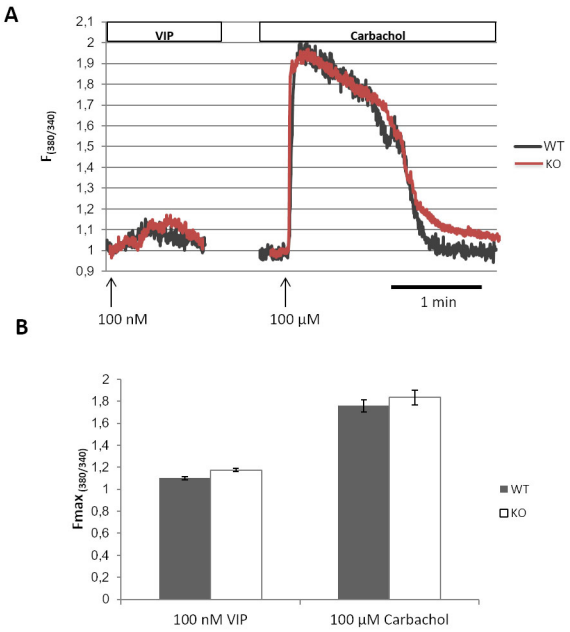

Figure 5. Effects of VIP and carbachol on $[Ca^{2+}]_i$ in lacrimal gland duct cells from WT and CFTR KO mice. **A**: Representative recordings of the microfluorescence experiments: Effects of vasoactive intestinal peptide (VIP; 100 nM) on $[Ca^{2+}]_i$ in lacrimal gland duct cells from wild-type (WT) and cystic fibrosis transmembrane conductance regulator (CFTR) knockout (KO) mice (panel **A**, left curve). Carbachol (100 µM) was used for comparison, as a positive control, in these experiments (panel **A**, right curve). $F_{380/340}$: 380/340 nm fluorescence emission ratio. **B**: Maximum values of the 380/340 nm fluorescence emission ratios. $F_{max(380/340)}$: maximum value of the 380/340 nm fluorescence emission ratio.

showed a reasonably homogeneous distribution in the duct system in contrast to the VPAC1 receptors [12,18,25]. A connection between CFTR and VPACs at the protein expression level also seems implausible, as the lack of CFTR did not influence the expression of VPAC1 and VPAC2 proteins: No difference was detected between the immunoreactivity of the WT and CFTR KO LG tissues in the experiments.

To explore the fluid secretory effect of VIP, videomicroscopic experiments were applied. VIP (100 nM) stimulation caused strong and continuous fluid secretion in the ducts from the WT mice. In contrast, as a result of VIP stimulation, the CFTR KO ducts exhibited only weak pulse-like secretion in the first 5 min, followed by a plateau. These findings are substantially different from the results we obtained previously in carbachol stimulated experiments where identical ductal fluid secretory responses were observed in the WT and CFTR KO mouse LG ducts [12]. The observed reduction in response to VIP stimulation in CFTR KO ducts may be explained by the lack of CFTR rather than changes in VPAC1 and VPAC2, as no difference was detected in the density of the VIP receptors between the WT and CFTR KO ducts in the present immunofluorescence studies. The role of CFTR can be explained by the following chain of effects: Stimulation of VIP receptors increases the intracellular cAMP levels via adenylyl-cyclase, and then cAMP (as a potent activator) increases the activity of CFTR. Therefore, this transporter seems to be the determining component of the robust fluid secretion evoked by VIP in WT ducts. The weak secretory response observed in the CFTR KO ducts during VIP stimulation seems to confirm the described mechanism.

Ca^{2+} homeostasis underlying VIP stimulation and the potential role of CFTR protein was also investigated. The effect of VIP stimuli on the cytosolic Ca^{2+} signaling was measured with microfluorometry. VIP acts not only through the adenylyl cyclase-cAMP system, but also, to a smaller extent, VIP elevates $[Ca^{2+}]_i$. VIP-evoked elevations of $[Ca^{2+}]_i$ were also independent from the presence of CFTR and did not differ in the WT and CFTR KO ducts in a significant manner. Therefore, VIP stimulation had a similar effect on $[Ca^{2+}]_i$ compared to the results of our previous experiments using carbachol stimulation. In those experiments, identical changes of $[Ca^{2+}]_i$ were found in WT and CFTR KO mouse LG ducts [12]. However, the elevations in $[Ca^{2+}]_i$ were much smaller in the case of VIP stimulation compared to the effect of carbachol. The role of these VIP-induced small changes of $[Ca^{2+}]_i$ was further investigated in fluid secretion experiments where intracellular Ca^{2+}-chelator BAPTA was applied. In a short series of preliminary experiments, WT ducts were incubated with BAPTA-AM (data not shown). No effect of the BAPTA-AM pretreatment on the robust VIP stimulated swelling response of WT ducts was detectable. The lack of demonstrability of the BAPTA-AM effect in the wild-type ducts can be explained by the sensitivity of the applied videomicroscopic method combined with the negligible effect of Ca^{2+}-dependent mechanisms in addition to the robust cAMP-dependent effect. In the next series of experiments, ducts isolated from CFTR KO mice were incubated with BAPTA before VIP stimulation. The suspended Ca^{2+} signaling completely abolished the remaining VIP-induced secretory response of mouse LG ducts lacking the cAMP-CFTR pathway. These results strongly suggest the minor complementary role of Ca^{2+}-regulated mechanisms in VIP-induced ductal fluid secretion. However, there is one detail that must be taken into consideration regarding these results: According to the mosaic pattern of VPAC1 receptors in the different duct segments, a remarkable portion of isolated ducts (approximately 50%–65% of all ducts in to complete series of all experiments) failed to react to VIP. This result suggests caution in the interpretation of negative results (i.e., non responding ducts). However, we performed a relatively high number of experiments in these series: eight ducts isolated from three different animals were investigated. We believe that this number makes our conclusions plausible and minimizes the probability of a random series of all non-responding ducts.

Findings in our previous experiments studying effects of carbachol stimulation and the present results in immunostaining studies and in fluid secretion together with $[Ca^{2+}]_i$ experiments suggest two partially independent parasympathetic regulatory pathways. Briefly, carbachol stimulation acts solely through elevation of $[Ca^{2+}]_I$, and it does not involve the adenylyl cyclase-cAMP route. Consequently, CFTR is not involved in carbachol-stimulated fluid secretion as it is activated by cAMP. For VIP stimuli, $[Ca^{2+}]_i$ was slightly elevated without a significant difference between the values measured in the WT and CFTR KO ducts. However, the secretory response of the LG ducts showed a large significant difference in the WT and CFTR KO ducts: the rate of fluid secretion of the CFTR KO ducts was far below the value obtained in the WT ducts. These data prove the determining role of the adenylyl cyclase-cAMP-CFTR route in VIP-stimulated fluid secretion of LG ducts. Results of the VIP experiments are in accordance with previous observations that VIP acts predominantly through elevation of the cytosolic cAMP level, and only a small part of the action of VIP is thought to be mediated by Ca^{2+} signaling [30,31]. The lack of functionally active CFTR seen in the CFTR KO mice influences the VIP-induced secretory response of LG ducts and thus, can modify

the total parasympathetic-evoked secretory contribution of the LG.

The observed VIP-induced significant fluid secretion suggests the importance of this neurotransmitter in the regulation of the duct system. LG duct cells have several different Cl^- and K^+ selective ion channels and transporters located on the basolateral and apical membranes. Elevated cytosolic cAMP levels and intracellular Ca^{2+} signaling can activate Cl^- and K^+ secretion through these channels and transporters. VIP seems to have target structures in basolateral and apical membranes. Previously, we reported the strong stimulatory effect of cell-permeable cAMP-analogue 8-bromo cAMP and VIP on basolaterally located sodium-potassium-chloride cotransporter type 1 (NKCC1) [32]. The present results suggest the important role of VIP in the regulation of apically located CFTR. Therefore, VIP seems to be able to activate Cl^- transport on the basolateral and apical sides of LG duct cells. In summary, actions of Cl^- selective channels located on the basolateral and apical membranes of duct cells result in intraluminal flux of Cl^-. Elevation of intraluminal Cl^- concentration is the main determinant of the lumen-negative transepithelial voltage difference, which is the driving force of ductal fluid secretion. Consequently, these results strongly suggest the important role of VIP in the regulation of lacrimal duct fluid secretion.

Identifying the mechanism underlying the regulation of ductal fluid transport is likely to be pertinent for treating ocular diseases. This study contributes to this objective by demonstrating that VIP-induced increases in cAMP followed by stimulation of CFTR chloride channel activity in the ductal epithelial cells drives fluid from the interstitium into the ductal lumen. This finding is relevant because treatment of some desiccating ocular surface diseases may benefit from applying cAMP mobilizing agents that, in turn, optimize this secretory process.

ACKNOWLEDGMENTS

Funding: The work was supported by the NKFIH NN 115611 (E T-M), the EFOP-3.6.1–16–2016–00008 (E T-M), the SZTE ÁOK-KKA No 2018/Tóth-MolnárE (E T-M), the NEI/NIH EY017731 (CD) and The Webb Foundation grant (CD).

REFERENCES

1. Alexander JH, van Lennep EW, Young JA. Water and electrolyte secretion by the exorbital lacrimal gland of the rat studied by micropuncture and catheterization techniques. Pflugers Arch 1972; 337:299-309. [PMID: 4674879].

2. Dartt DA, Moller M, Poulsen JH. Lacrimal gland electrolyte and water secretion in the rabbit: localization and role of (Na^+-K^+)-activated ATPase. J Physiol 1981; 321:557-69. [PMID: 6461755].

3. Ubels JL, Hoffman HM, Srikanth S, Resau JH, Webb CP. Gene expression in rat lacrimal gland duct cells collected using laser capture microdissection: evidence for K^+ secretion by the duct cells. Invest Ophthalmol Vis Sci 2006; 47:1876-85. [PMID: 16638994].

4. Tóth-Molnár E, Venglovecz V, Ozsvári B, Rakonczay Z, Varró A, Papp JG, Tóth A, Lonovics J, Takáts T, Ignáth I, Iványi B, Hegyi P. New experimental method to study acid/base transporters and their regulation in lacrimal gland ductal epithelia. Invest Ophthalmol Vis Sci 2007; 48:3746-55. [PMID: 17652747].

5. Katona M, Vizvári E, Németh L, Facskó A, Venglovecz V, Rakonczay Z, Hegyi P, Tóth-Molnár E. Experimental evidence of fluid secretion of lacrimal gland duct epithelium. Invest Ophthalmol Vis Sci 2014; 55:4360-7. [PMID: 24925876].

6. 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 Sci 1997; 38:610-9. [PMID: 9071214].

7. Said SI, Mutt V. A peptide fraction from lung tissue with prolonged peripheral vasodilator activity. Scand J Clin Lab Invest Suppl 1969; 107:51-6. [PMID: 5375727].

8. Gilbard JP, Dartt DA, Rood RP, Rossi SR, Gray KL, Donowitz M. Increased tear secretion in pancreatic cholera: a newly recognized symptom in an experiment of nature. Am J Med 1988; 85:552-4. [PMID: 2845780].

9. Laburthe M, Couvineau A, Tan V. Class II G protein-coupled receptors for VIP and PACAP: structure, models of activation and pharmacology. Peptides 2007; 28:1631-9. [PMID: 17574305].

10. Laburthe M, Couvineau A, Marie JC. VPAC receptors for VIP and PACAP. Receptors Channels 2002; 8:137-53. [PMID: 12529932].

11. Laburthe M, Couvineau A. Molecular pharmacology and structure of VPAC Receptors for VIP and PACAP. Regul Pept 2002; 108:165-73. [PMID: 12220741].

12. Berczeli O, Vizvári E, Katona M, Török D, Szalay L, Rárosi F, Németh I, Rakonczay Z, Hegyi P, Ding C, Tóth-Molnár E. Novel Insight into the Role of CFTR in Lacrimal Gland Duct Function in Mice. Invest Ophthalmol Vis Sci 2018; 59:54-62. [PMID: 29305607].

13. Hong JH, Park S, Shcheynikov N, Muallem S. Mechanism and synergism in epithelial fluid and electrolyte secretion. Pflugers Arch 2014; 466:1487-99. [PMID: 24240699].

14. Lee MG, Ohana E, Park HW, Yang D, Muallem S. Molecular mechanism of pancreatic and salivary gland fluid and HCO3 secretion. Physiol Rev 2012; 92:39-74. [PMID: 22298651].

15. Reddy MM, Quinton PM. PKA mediates constitutive activation of CFTR in human sweat duct. J Membr Biol 2009; 231:65-78. [PMID: 19865788].

16. Castagna I, Roszkowska AM, Fama F, Sinicropi S, Ferreri G. The eye in cystic fibrosis. Eur J Ophthalmol 2001; 11:9-14. [PMID: 11284496].

17. Sheppard JD, Orenstein DM, Chao CC, Butala S, Kowalski RP. The ocular surface in cystic fibrosis. Ophthalmology 1989; 96:1624-30. [PMID: 2616148].

18. Lu M, Ding C. CFTR-mediated Cl- transport in the acinar and duct cells of rabbit lacrimal gland. Curr Eye Res 2012; 37:671-7. [PMID: 22578307].

19. Frizzell RA, Hanrahan JW. Physiology of epithelial chloride and fluid secretion. Cold Spring Harb Perspect Med 2012; 2:a009563-[PMID: 22675668].

20. Hwang TC, Kirk KL. The CFTR ion channel: gating, regulation, and anion permeation. Cold Spring Harb Perspect Med 2013; 3:a009498-[PMID: 23284076].

21. Tóth-Molnár E, Berczeli O, Vizvári E, Rakonczay Z, Hegyi P, Ding C. Effect of vasoactive intestinal peptide on lacrimal gland ductal fluid secretion in wild type and CFTR knockout mice. Invest Ophthalmol Vis Sci 2017; 58:2242-.

22. Ratcliff R, Evans MJ, Cuthbert AW, MacVinish LJ, Foster D, Anderson JR, Colledge WH. Production of a severe cystic fibrosis mutation in mice by gene targeting. Nat Genet 1993; 4:35-41. [PMID: 7685652].

23. Seidler U, Singh A, Chen M, Cinar A, Bachmann O, Zheng W, Wang J, Yeruva S, Riederer B. Knockout mouse models for intestinal electrolyte transporters and regulatory PDZ adaptors: new insights into cystic fibrosis, secretory diarrhoea and fructose-induced hypertension. Exp Physiol 2009; 94:175-9. [PMID: 18931049].

24. Fernández-Salazar MP, Pascua P, Calvo JJ, Lopez MA, Case RM, Steward MC, San Roman JI. Basolateral anion transport mechanisms underlying fluid secretion by mouse, rat and guinea-pig pancreatic ducts. J Physiol 2004; 556:415-28. [PMID: 14978209].

25. Ding C, Parsa L, Nandoskar P, Zhao P, Wu K, Wang Y. Duct system of the rabbit lacrimal gland: structural characteristics and role in lacrimal secretion. Invest Ophthalmol Vis Sci 2010; 51:2960-7. [PMID: 20107177].

26. Ding C, Lu M, Huang J. Changes of the ocular surface and aquaporins in the lacrimal glands of rabbits during pregnancy. Mol Vis 2011; 17:2847-55. [PMID: 22128232].

27. Thomson-Vest N, Shimizu Y, Hunne B, Furness JB. The distribution of calcium-activated, intermediate conductance potassium (IK) channels in epithelial cells. J Anat 2006; 208:219-29. [PMID: 16441566].

28. Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. Prog Retin Eye Res 2009; 28:155-77. [PMID: 19376264].

29. Dartt DA. Signal transduction and control of lacrimal gland protein secretion: a review. Curr Eye Res 1989; 8:619-36. [PMID: 2545411].

30. Dérand R, Montoni A, Bulteau-Pignoux L. Activation of VPAC1 receptors by VIP and PACAP-27 in human bronchial epithelial cells induces CFTR-dependent chloride secretion. Br J Pharmacol 2004; 141:698-708. [PMID: 14744818].

31. Dickson L, Aramori I, McCulloch J. A systematic comparison of intracellular cyclic AMP and calcium signalling highlights complexities in human VPAC/PAC receptor pharmacology. Neuropharmacology 2006; 51:1086-98. [PMID: 16930633].

32. Vizvári E, Katona M, Orvos P, Berczeli O, Facskó A, Rárosi F, Venglovecz V, Rakonczay Z, Hegyi P, Ding C, Tóth-Molnár E. Characterization of Na^+-K^+-$2Cl^-$ Cotransporter Activity in Rabbit Lacrimal Gland Duct Cells. Invest Ophthalmol Vis Sci 2016; 57:3828-35. [PMID: 27438543].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 6 December 2020. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.