The role of parasympathetic regulation in the fluid secretion of lacrimal gland ducts in mice

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LIST OF RELATED FULL PAPERS

The thesis is based on the following publications:

1. Novel Insight Into the Role of CFTR in Lacrimal Gland Duct Function in Mice.
   INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE 2018; 59(1):54-62. (2018)

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2. Characterization of Na^+-K^+-$2Cl^-$ Cotransporter Activity in Rabbit Lacrimal Gland Duct Cells.
   INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE 2016; 57(8): 3828-3835.
   Vizvari E, Katona M, Orvos P, **Berczeli O**, Facsko A, Rarosi F, Venglovecz V, Rakonczay Z Jr, Hegyi P, Ding C, Tóth-Molnár E.

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1. INTRODUCTION

The ocular surface including the cornea and conjunctiva and its overlying tear film is the first barrier of the eye to interact with the external environment. The lack of appropriate tear production may result in dry eye and the potential for significant surface pathology. Lacrimal gland (LG) contributes to the production of tear with several components. This process is mediated by an array of ion transporters/channels. More detailed understanding of the physiology and pathophysiology of LG can lead us to find pharmacological targets of dry eye disease. LG is a tubuloacinar exocrine gland and composed mainly of 3 types of cells: acinar, ductal and myoepithelial cells. Acinar cell is giving approximately 80% of all cells in the gland. Duct cells make up to 15% of all cells in LG forming the duct system and modify the secretory product as it moves through the ducts. LG is innervated by both the parasympathetic and sympathetic nerves. The major neurotransmitters that regulate secretion are the parasympathetic neurotransmitters acetylcholine and vasoactive intestinal peptide (VIP).

Acinar cell functions are widely studied, resulting in broad spectrum of information, however much less is known about the possible secretory function of duct cells. In 1972, Alexander and coworkers were among the first pioneers who proposed the potential role of duct system in LG secretion. They suggested that tears are formed in two distinct stages: the plasma-like primary fluid formed by acinar cells is modified by duct cells during its transit in the duct lumen. Modifications of K^+ and Cl^- content of primary acinar fluid by ductal secretory processes were also proposed in 1981 by Dartt and colleagues. However, the role of LG duct epithelium on fluid, electrolyte, and protein secretion was not well understood at that time.

The first experimental model for the investigation of lacrimal duct function was adapted and applied by our group. Preparation of isolated intact LG ducts enabled the performance of real-time functional experiments on LG ducts for the first time. By a video-microscopic technique adapted and used for the first time in lacrimal duct research we investigate fluid secretion of isolated LG duct segments. Our previous experimental results gave evidence of the active role of the duct LG system in lacrimal fluid secretion.

Although earlier reports demonstrated the contribution of VIP released by parasympathetic nerves in acinar protein secretion, its impact on the fluid secretion of the LG ducts remained unknown. The VIP-receptor interaction activates the G protein $G_s\alpha$ that stimulates adenylyl cyclase, that increases intracellular cAMP level. CAMP in turn activates

protein kinase A (PKA) that stimulates secretion by phosphorylating target proteins. Hormones and neurotransmitters, such as VIP, which raise cellular cAMP level, can stimulate acute Cystic fibrosis transmembrane conductance regulator (CFTR) channel activity. Our findings - based on indirect evidences - strongly suggested the role of CFTR in ductal fluid secretion as forskolin - a well-known activator of CFTR via the elevation of cytosolic cAMP levels – resulted in a significant swelling response in our experimental setup. Furthermore, there are several clinical data about dry eye disease with various severity seen in CF patients, which implies the potential influence of CFTR in altered tear secretion. Accumulating evidences from gene expression studies performed on rat and rabbit LGs demonstrated the predominant expression of CFTR in LG duct cells. The main role of the CFTR is a regulated anion conductance in the apical membrane of many different epithelial cell types. Regulation of channel activity is predominantly via cAMP/PKA signaling. Availability of transgenic mouse models carrying genetic defects in CFTR allows us the direct examination of its role in LG.

2. AIMS OF THE STUDY

LG is the major source of tears that bathe the ocular surface and its secretion is dominantly mediated by the neurotransmitters released by the parasympathetic nerves, like acetylcholine and VIP. Although earlier reports demonstrated the presence of cholinergic and VIP receptors on LG cells and also their contribution in acinar protein secretion, the impact of parasympathetic transmitters on the fluid secretion of LG ducts is completely unknown. Therefore we aimed to investigate the role of parasympathetic stimulation induced by cholinomimetic compound carbachol or VIP on LG duct function in mouse.

CFTR plays a critical role in the transmembrane transport of chloride in many secretory epithelia. There are accumulating evidences that VIP stimulation and CFTR functions are tightly related and regulated. Therefore investigation of the role of CFTR in the parasympathetic secretory machinery in mouse LG ducts was also aimed.

Our specific aims were:

- To explore the role of cholinergic stimulation on fluid secretion in LG ducts isolated from wild type (WT) and CFTR knockout (KO) mice.

- To investigate fluid secretion of isolated LG duct segments evoked by VIP in WT and CFTR KO LG ducts.
- To investigate the Ca^{2+} homeostasis underlying carbachol and VIP stimulation both in WT and CFTR KO LG ducts.

3. METHODS

Animals

LG ducts were isolated from adult wild type and CFTR knock-out mice. All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols 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.

H&E staining and immunofluorescence

Dissected LG tissues, from both WT and KO mice, were fixed, cut and stained with H&E. Cryostat sections were rehydrated, then fixed. Following overnight incubation with primary antibodies the samples were incubated with secondary antibody for 1 hour.

Isolation and culture of lacrimal duct segments

LGs were dissected and following incubation steps the tissue pieces were transferred to a glass microscope slide and viewed under stereo microscope. Inter-, and intralobular ducts were micro-dissected and after microdissection, intact LG ducts were transferred to the culture solution in a Petri dish. Ducts were cultured overnight.

Measurement of fluid secretion of LG ducts

Videomicroscopic technique was used for measurement of ductal fluid secretion. Fluid secretion into closed intraluminal space of cultured lacrimal gland interlobular ducts was analyzed with the swelling method using an inverted microscope.

Measurement of intracellular Ca^{2+} level ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured using Ca^{2+}-sensitive fluorescent dye FURA 2AM. Small areas (ROIs) of 5 to 10 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 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 ANOVA model was used for the calculation of ductal fluid secretion. Kruskal-Wallis test with Dunn method was used for the analysis of tear secretion. SPSS 22 statistical software (IBM, New York, USA) was used to analyze the data, which were presented as means ± SEM. A p value of less, than 0.05 was regarded as significant.

4. RESULTS

Hematoxylin and eosin (H&E) staining of LGs from WT and CFTR KO mice

The morphologic differences between LGs originated from KO and WT mice were investigated by H&E staining. Freshly dissected LGs of 8 – 10 and 20 – 24 week-old animals were processed. No obvious structural changes have been observed in KO LG tissues as compared to their age-matched WT counterparts.

Immunofluorescence

Immunofluorescence was used to confirm the presence of VPAC1, VPAC2 receptors and CFTR in mouse LG duct cells. We proved the presence of CFTR protein most prominently in the apical membranes of LG duct cells from WT animals, although some diffuse staining was also found in acinar cells, mostly within the cytoplasm. As anticipated, we were unable to detect the presence of CFTR protein in LGs originated from KO mice. VPAC1 is dominant in the duct cells. Immunofluorescence staining of VPAC1 receptors revealed a mosaic pattern in the expression of the receptor proteins No difference was detected in the immunoreactivity between LG tissues of WT and KO mice. Intense VPAC2 staining was detected not only in the duct cells but also in the basolateral surface of the acinar cells. Intensity of immunofluorescence staining was similar in case of both WT and KO mice.

Cholinergic-stimulated fluid secretion in isolated LG ducts

To investigate the role of CFTR in cholinergic-evoked ductal fluid secretion, the responses to muscarinic agonist carbachol were examined in WT and KO LG ducts both in HEPES and HCO_3^-/CO_2-buffered solution. Duct segments were isolated from 14 to 24-week-old mice. Rapid secretion could be observed in the first 4-5 minutes of carbachol stimulation followed by a slower phase in HEPES buffered solution. Carbachol-evoked fluid secretory rates observed in WT and in KO ducts did not differ significantly in HEPES-buffered

solution. Carbachol-evoked fluid secretion in WT and in KO ducts superfused with HCO_3^-/CO_2-buffered solution was then investigated. Similarly to the results obtained in HEPES buffer a rapid secretory effect could be detected in the first 4-5 min both in WT and in KO ducts followed by slower secretion. Carbachol-evoked fluid secretion observed in WT and in KO ducts did not differ significantly in HCO_3^-/CO_2-buffered solution. Briefly, carbachol stimulated fluid secretion in a similar manner both in WT and KO ducts and both in HEPES and HCO_3^-/CO_2-buffered solutions. There was not significant difference observed in secretory rates either between WT and KO ducts or between different buffers.

VIP-induced fluid secretion in isolated LG ducts

Effect of VIP on ductal fluid secretion was investigated on both WT and CFTR KO mouse LG ducts. These experiments were performed exclusively in HCO_3^-/CO_2-buffered solutions. Motives of solely HCO_3^-/CO_2 buffered experiments were partly the identical results obtained in different (HEPES and HCO_3^-/CO_2-) buffered solution in our previous experiments performed on different species. Furthermore, our VIP experiments were restricted to the HCO_3^-/CO_2 buffered solutions because of the difficulties observed during our VIP-stimulated fluid secretion experiments. In these experiments remarkable portion of isolated ducts missed to react to the applied agent. These findings are in accordance with our results in VPAC1 immunofluorescence studies where the expression of the receptor protein showed a mosaic pattern. VIP stimulation resulted in a robust and continuous fluid secretory response in isolated LG duct segments originated from WT mice. In contrast, CFTR KO ducts exhibited only a very weak pulse-like secretion in the first 5 minutes of stimulation, followed by a plateau.

Carbachol and VIP-induced Ca^{2+} signaling of isolated LG ducts

To investigate the effect of carbachol and the potential difference in $[Ca^{2+}]_i$ between WT and KO ducts, carbachol-evoked elevation of $[Ca^{2+}]_i$ was measured in both groups of isolated ducts. $[Ca^{2+}]_i$ was elevated by carbachol in a dose dependent manner both in WT and in KO duct cells. No significant differences between WT and KO ducts were observed in these experiments. Intracellular Ca^{2+} homeostasis underlying VIP stimulation was measured both in WT and in CFTR KO duct segments. VIP stimulation resulted in a small, but statistically significant increase in $[Ca^{2+}]_i$ both in WT and in CFTR KO ducts. Cholinergic agonist carbachol was used as positive control in these experiments.

5. DISCUSSION

Dry eye disease is an increasing health care problem in the industrialized countries affecting millions of people worldwide. Most of the available treatments focus on the alleviation of ocular symptoms, without targeting its underlying cause. Appropriate amount of balanced electrolyte, protein and mucin composition of fluid secreted by the LG is fundamental for maintaining preocular tear film volume and integrity. More detailed understanding of LG function is therefore essential in order to develop novel pharmacological approaches in the treatment of dry eye disease. Unfortunately, duct cells have been understudied for many years compared to the widely investigated acinar cells. Our laboratory developed the first experimental model suitable for the investigation of lacrimal duct function, which opened a new way 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.

Our knowledge about the regulation of LG duct function is rather limited. LG duct secretion is mediated by both parasympathetic and sympathetic systems. 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 LG secretion. The available experimental evidences about the role of the paraysmpathetic system in LG fluid secretion are solely from intact LGs or acini. We investigated the direct effect of the cholinergic and VIP-ergic stimuli on isolated LG duct segments, and explored the relationship between the CFTR chloride channel and the parasympathetic regulatory system. Transgenic mouse models carrying genetic defects in CFTR made it possible to set up experiments for investigating its role in fluid secretion and to shed light on the potential morphological alterations caused by the missing CFTR protein.

To assess the potential morphologic differences between LGs from CFTR KO and WT mice, histological examinations were performed on freshly dissected LGs. H&E staining revealed no obvious structural changes in CFTR KO LG tissues including ducts as compared to their age-matched WT counterparts. These results demonstrated that functional failure may present before morphological alterations during the course of disease progression. However, investigation of the morphological alterations in later stages of the disease in CFTR KO mice has strong limitations as shorter life expectancies of CFTR KO mice. Further studies are needed in order to clarify potential alterations in LG morphology later in life.

Immunofluorescence was used to confirm the presence and localization of CFTR, VPAC1 and VPAC2 proteins of mouse LG duct cells. We could observe intense CFTR staining most characteristically in the apical membranes of LG duct cells from WT animals. Some substantially weaker diffuse staining was also found in acinar cells, mostly within the cytoplasm. Although the appearance of CFTR protein is undoubted in acinar cells of mouse LG, our immunostaining results pointed its predominant presence in duct cells. These results suggest the important and duct-specific function of CFTR in LG similarly to its role in pancreatic ducts. According to our expectations CFTR protein was not detectable in LGs of CFTR KO mice.

Presence and localization of VPAC1 and VPAC2 receptors was also investigated in LGs of both WT and CFTR KO mice. An apparent difference was revealed in the distribution of VPAC1 and VPAC2 receptors in mouse LG in our study. Presence of VPAC1 was proved predominantly in ducts by immunoreactivity. Beside the characteristically ductal localization of VPAC1 receptors, a mosaic pattern in the expression of the receptor proteins was also revealed. Intensity of immunofluorescence varied excessively in the analyzed duct segments from the intense staining to the almost complete lack of demonstrability. VPAC2 showed a more homogeneous distribution in LG tissue: these receptors were traceable in both acinar and duct cells. Both VPAC1 and VPAC2 were observed most prominently on the basolateral membrane of mouse LG cells. Lack of CFTR did not influence the expression of VPAC1 and VPAC2 proteins: no difference was detected between the immunoreactivity of WT and CFTR KO LG tissues.

To explore the fluid secretory effect of parasympathetic neurotransmitters, videomicroscopic experiments were applied. Cholinergic agonist carbachol (100 μM) caused a biphasic fluid secretory response consisting of a rapid pulse-like secretion in the first 5 min, followed by a plateau. Similar secretory rates and patterns were observed in KO ducts, suggesting the independence of cholinergic activated secretory mechanisms from the presence of functionally active CFTR. VIP (100 nM) stimulation caused a strong and continuous fluid secretion in ducts from WT mice. In contrast, as a result of VIP stimulation, CFTR KO ducts exhibited only a weak pulse-like secretion in the first 5 min, followed by a plateau. The observed reduction in response to VIP stimulation in CFTR KO ducts may be explained by the lack of CFTR rather than changes of VPAC1 and VPAC2 since no difference was detected in the densitiy of VIP receptors between WT and CFTR KO ducts in our immunofluorescence studies. Role of CFTR can be explained by the following chain of effects: stimulation of VIP receptors increases the intracellular cAMP levels via adenylyl-

cyclase 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 CFTR KO ducts during VIP stimulation seems to confirm the described mechanism.

Ca^{2+} homeostasis underlying parasympathetic stimulation and the potential role of CFTR protein was also investigated. The effect of carbachol and VIP stimuli on the cytosolic Ca^{2+} signaling was measured by microfluorometry. Carbachol induced a dose-dependent elevation of $[Ca^{2+}]_i$ in both WT and KO duct cells. The carbachol-evoked response of cytosolic Ca^{2+} level changes in WT and CFTR KO ducts did not differ significantly. The unchanged carbachol-evoked elevation of $[Ca^{2+}]_i$ in parallel with the unchanged carbachol-evoked secretory response in CFTR KO duct cells suggests the determining or exclusive role of Ca^{2+} signaling in cholinergic stimulation and excludes the considerable function of CFTR in this pathway. VIP also acts not only through the adenylyl cyclase-cAMP system but – in a smaller extent – it also elevated $[Ca^{2+}]_i$. VIP-evoked elevations of $[Ca^{2+}]_i$ were also independent from the presence of CFTR and did not differ in WT and CFTR KO LG ducts in a significant manner. However, elevations of $[Ca^{2+}]_i$ had a much smaller extent in case of VIP stimulation compared to the carbachol effect.

Our findings in immunostaining studies and in fluid secretion together with $[Ca^{2+}]_i$ experiments suggests two, partially independent parasympathetic regulatory pathways. Briefly, carbachol stimulation acts solely through elevation of $[Ca^{2+}]_i$ and it does not involve 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 significant difference between the values measured in WT and CFTR KO ducts. However, secretory response of LG ducts showed a great significant difference in WT and CFTR KO ducts: rate of fluid secretion of CFTR KO ducts was far below the value obtained in WT ducts. These data prove the determining role of adenylyl cyclase-cAMP-CFTR route in VIP-stimulated fluid secretion of LG ducts. Results of our VIP experiments are in accordance with previous observations that VIP acts predominantly through elevation of cytosolic cAMP level, the minority of its action thougt to be mediated by Ca^{2+} signaling. Lack of functionally active CFTR seen in CFTR KO mice influences VIP-evoked secretory response of LG ducts and thus can modify the total parasympathetic-evoked secretory answer of the LGs. CFTR could influence LG secretion through the modification of ductal secretion and therefore defects in CFTR may significantly compromise Cl^- and water secretion from LG ducts.

6. SUMMARY

In conclusion, our results presented here provide new proof of the secretory role of LG ducts beside their piping function. We demonstrated the functional presence of CFTR and VIP receptors in LG duct cells. Strong predominance of CFTR protein on the apical, VPAC1 and VPAC2 on the basolateral surface of the duct cells was observed. Fluid secretory experiments provided direct functional evidence of the importance of parasympathetic regulation in LG ducts. We detected the elevation $[Ca^{2+}]_i$ for the stimuli of carbachol an VIP, and the preservation of the Ca^{2+} signaling pathway in CFTR KO mice. These results reveal new insight into the role of VIP in LG function and confirm the connection between CFTR and VIP regulatory pathways. Further studies are needed to clarify whether modification of CFTR or VIP function may serve as a potential target to stimulate LG secretion and therefore can be an option in treating aqueous deficient dry eye.

Conclusions of the studies on LG ducts presented in the thesis are:

1. **CFTR is dominantly expressed on the apical membrane of the LG duct cells compared to acinar cells in mice**
2. **VPAC1 and VPAC2 is expressed on the basolateral membrane of the LG duct and acinar cells in mice**
3. **For the first time in LG research, CFTR KO mice were used to study the role of parasympathetic regulation in LG duct function and to study the role of CFTR in the parasympathetic secretory machinery**
4. **Using our isolated duct segment model developed earlier, fluid secretion of LG ducts from WT and CFTR KO mice was investigated by videomicroscopic methods. Significant fluid secretion was observed as a result of carbachol and VIP stimulation.**
5. **The significant secretory response of LG ducts from KO mice for carbachol stimuli showed the independence of cholinergic activated secretory mechanisms from the presence of functionally active CFTR.**
6. **Role of CFTR in the VIP-induced secretory process found to be decisive as only small VIP-induced fluid secretion was detected in the absence of CFTR protein.**
7. **Similar elevations of $[Ca^{2+}]_i$ levels in WT and KO ducts - both in case of carbachol and VIP stimuli - demonstrated the independence of $[Ca^{2+}]_i$ homeostasis from the functionally active CFTR.**

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