Role of cystic fibrosis transmembrane conductance regulator in lacrimal gland secretion

Final Report

OTKA NN115611

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The aim of our project was to investigate the role of cystic fibrosis transmembrane conductance regulator (CFTR) in fluid secretion of lacrimal gland. Regulation of fluid secretion and interactions of CFTR with other transporters were also investigated in our studies.

A unique new isolation technique was developed by our research group prior to the present project. Function of ductal epithelial cells can be studied with the help of this model: changes of intracellular pH and Ca^{2+} levels can be detected by microfluorophotometry and presence and role of different basolateral transmembrane transporters can be investigated [Tóth-Molnár et al., Invest Ophthalmol Vis Sci 2007; 48: 3746-3755.2007]. The capability of fluid secretion of ductal epithelial cells was also proved by our group using the videomicroscopic swelling method [Katona et al., Invest Ophthalmol Vis Sci 2014; 55: 4360-4367].

Studies of the project

1) *In vivo* experiments to investigate tear secretion and ocular surface alterations

Tear secretion was measured using cotton threads in different age groups (at 8 to 10 weeks of age and at 20 to 24 weeks of age) both in wild type (WT) and CFTR KO animals. Significant decrease in tear secretion was observed in CFTR KO mice in every age group compared to the WT littermates (Figure 1). These secretion studies further suggest the critical role of CFTR may play in lacrimal production.

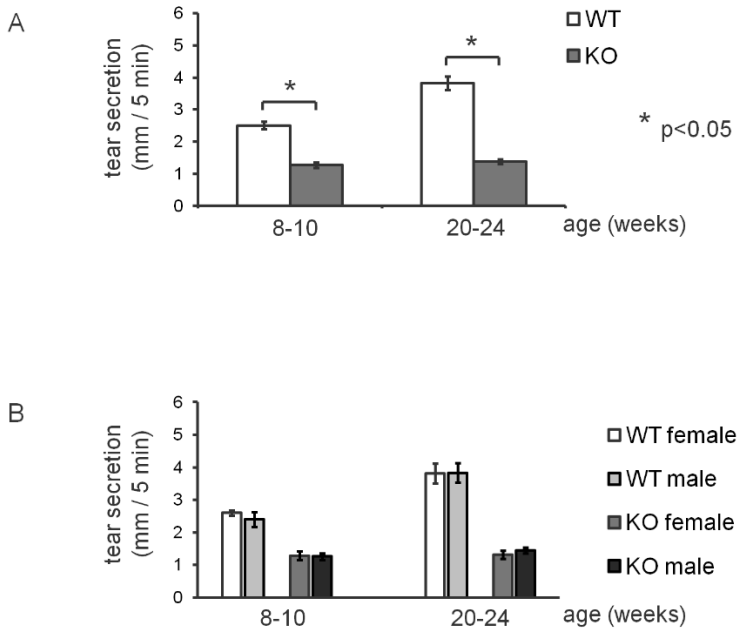

Figure 1. Tear secretions of WT and KO mice in 2 age groups.
A) Tear secretion was measured in two age groups in both WT and CFTR KO mice: at 8-10 weeks of age and at 20-24 weeks of age. Data from both eyes were averaged and evaluated. Tear secretion of CFTR KO mice were significantly lower compared to WTs in both age groups.
B) Secretion results of male and female mice were assessed and evaluated separately in WT and KO animals in both age groups. Gender-based data did not show significant difference in tear secretion either in WT or in KO mice. Data are presented as means±SEM.

To evaluate ocular surface integrity, fluorescein staining was performed also at 8 to 10 weeks of age and at 20 to 24 weeks of age on both WT and CFTR knockout mice by applying 0.5 µL Na^+-fluorescein into the inferior conjunctival sac. The ocular surface was investigated using cobalt blue filtered slit-lamp, and the images were evaluated and graded. Corneal staining score was higher in CFTR KO animals in each age group compared to the WT littermates (Figure 2).

Reduced tear secretion and increased corneal fluorescein staining results observed in CFTR KO mice clearly demonstrate that lack of CFTR deeply impairs the integrity of the ocular surface. Global dysfunction of the lacrimal functional unit could be observed when CFTR protein is absent in these mice.

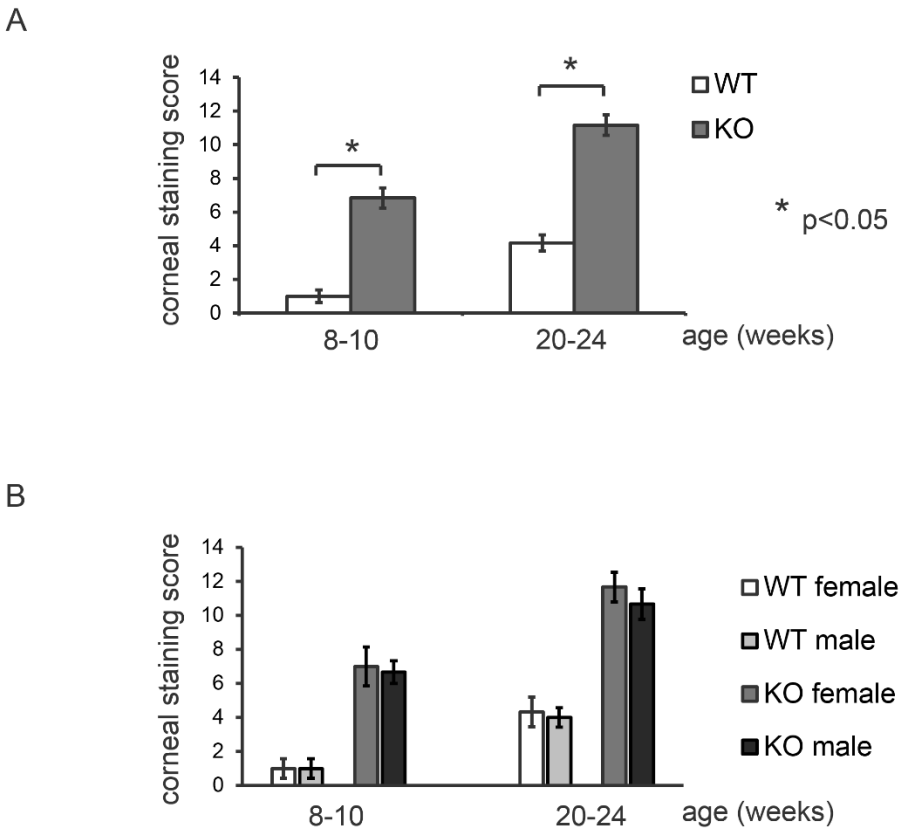

Figure 2. Corneal fluorescein staining in WT and CFTR KO mice.
A) Corneal fluorescein staining was measured in two age groups: at 8-10 weeks of age and at 20-24 weeks of age in both WT and CFTR KO mice. Data from both eyes were averaged and evaluated. Corneal staining scores of KO mice were significantly higher compared to WTs in both age groups.
B) Corneal fluorescein staining scores of male and female mice were calculated separately in WT and KO animals in both age groups. No significant differences were found between males and females either in WT or in KO mice. Data are presented as means±SEM.

2) <u>Immunofluorescence localization of CFTR in mouse lacrimal gland</u>

Immunofluorescence staining was used for localization of CFTR in lacrimal gland cells. As demonstrated in Figure 3 (panel A and B), intense CFTR staining could be found most prominently in the apical membranes of lacrimal gland 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 lacrimal glands originated from KO mice (Figure 3 panel C and D).

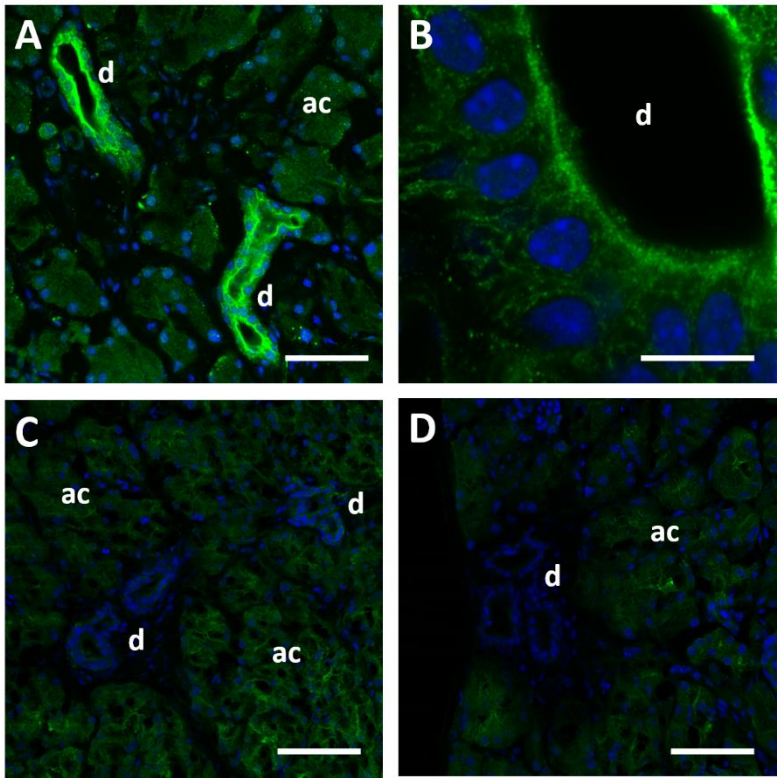

Figure 3. Immunofluorescence staining of CFTR in lacrimal glands from WT and CFTR KO mice. In lacrimal glands from WT mice (A, B), CFTR staining was most prominent in the apical membranes of duct cells (d), compared to acinar cells (ac). No staining could be observed in lacrimal glands from KO mice (C, D). DAPI was used to stain nuclei as blue. Scale bar in A, C and D, 50 μm; in B, 10 μm.

2) Fluid secretion and Ca^{2+} homeostasis of isolated lacrimal gland duct segments

Effects of both forskolin and carbachol on the secretory response of isolated duct segments deriving from WT and CFTR KO mice were investigated in videomicroscopic secretion studies. Interlobular and intralobar duct segments were isolated from lacrimal gland of WT and KO mice. Intracellular cAMP elevation i.e. forskolin stimulation resulted in a rapid and sustained secretory response from WT mice whereas no secretory response could be detected in CFTR KO animals (Figure 4). These results further corroborate the critical role of CFTR plays in lacrimal gland function. Role of CFTR in the cholinergic-evoked ductal fluid secretion was also investigated. Cholinergic agonist carbachol caused a biphasic secretory response consisting of a rapid pulse-like secretion in the first 5 min, followed by a plateau phase with similar secretory patterns both in WT and in CFTR KO ducts (Figure 5).

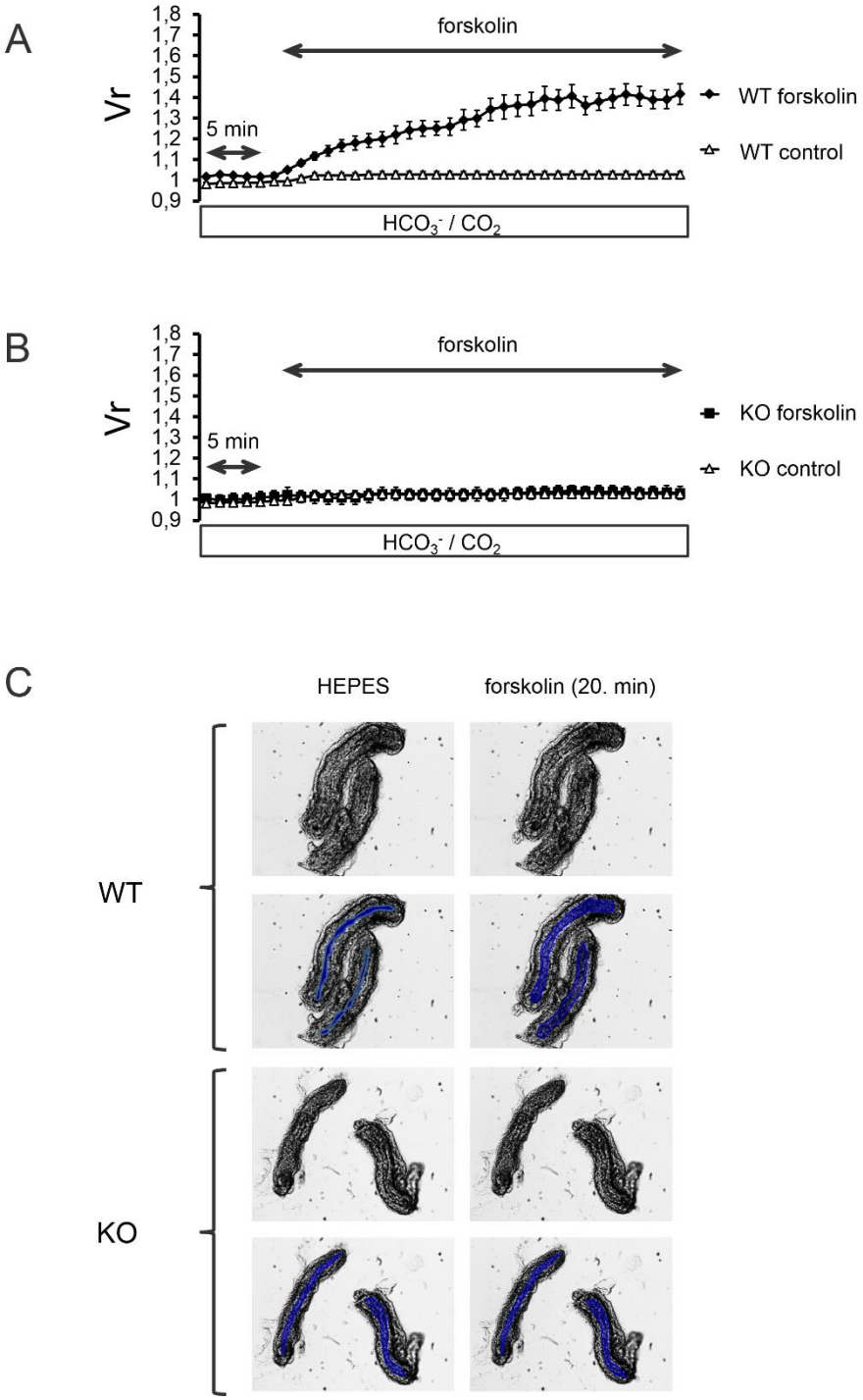

Figure 4. Effect of forskolin on fluid secretion in mouse ducts isolated from WT and CFTR KO lacrimal glands. A) WT ducts were exposed either to 10 μM forskolin (filled rhombus) or to no agonist (empty triangle). B) CFTR KO ducts were exposed either to 10 μM forskolin (filled square) or to no agonist (empty triangle). Changes in relative luminal volume (Vr) are shown. Data were obtained from 6 ducts isolated from 3 different animals in each series and are presented as means ± SEM. C) Photo series of secreting isolated lacrimal gland duct segments in response to forskolin stimulation. The secretory responses observed in HEPES-buffered and in HCO_3^-/CO_2-buffered solutions were similar. The luminal space is marked with blue color.

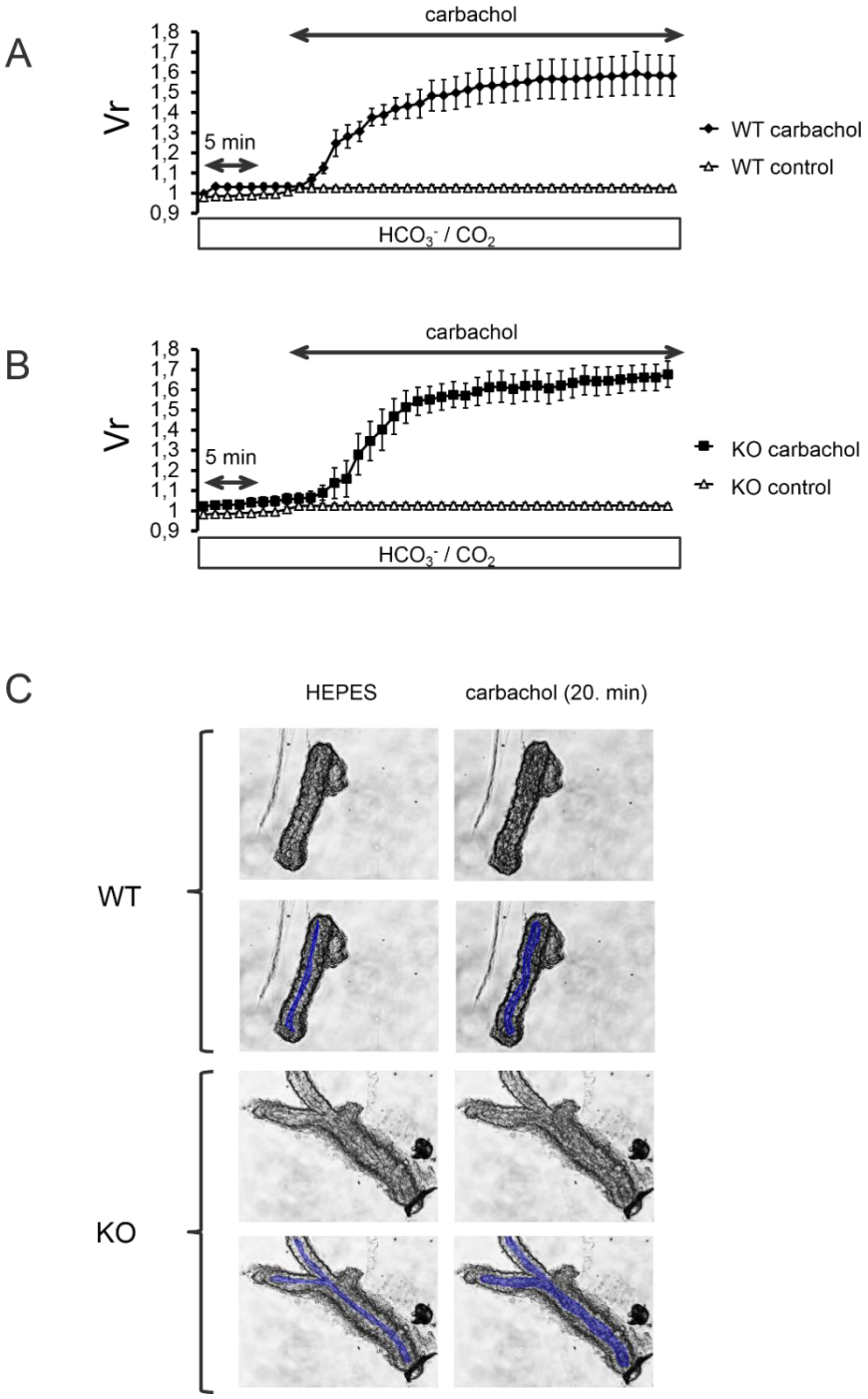

Figure 5. Effect of carbachol on ductal fluid secretion in mouse ducts isolated from WT and CFTR KO lacrimal glands. A) WT ducts were exposed either to 100 µM carbachol (filled rhombus) or to no agonist (empty triangle). B) KO ducts were exposed either to 100 µM carbachol (filled square) or to no agonist (empty triangle). Changes in relative luminal volume (Vr) are shown. Data were obtained from 6 ducts isolated from 3 different animals in each series and are presented as means ± SEM. C) Photo series of secreting isolated lacrimal gland duct segments in response to carbachol stimulation. The secretory responses observed in HEPES-buffered and in HCO_3^-/CO_2-buffered solutions were similar. The luminal space is marked with blue color.

Intracellular Ca^{2+} homeostasis underlying cholinergic stimulation was also investigated in ducts from WT and CFTR KO mice. Intracellular Ca^{2+} level was measured with microfluorometry and Ca^{2+}-sensitive fluorescent dye FURA 2AM using three different doses of carbachol. Carbachol dose-dependently elevated the cytosolic Ca^{2+} level without significant difference between the ducts from WT and CFTR KO mice.

The complete absence of forskolin-stimulated fluid secretion observed in CFTR KO ducts represents the decisive role of CFTR may play in lacrimal gland duct secretion in mice. Secretion of mouse lacrimal gland ducts found to be a Cl^--driven process similarly to our earlier findings with the rabbit lacrimal gland ducts. Carbachol-evoked fluid secretion proved to be the same in WT and CFTR KO ducts suggesting the independence of secretory mechanisms activated by Ca^{2+} mobilizing stimuli from functionally active CFTR.

3) Characterization of Na^+-K^+-2Cl^- cotransporter 1 (NKCC1) activity

Our results proved that secretion of mouse lacrimal gland ducts is a Cl^--driven process and CFTR plays an important role in Cl^- transport on the apical side of lacrimal gland duct cells. To investigate the Cl^- transport on the basolateral side of duct cells we studied the role of NKCC1. This transporter has an important function in the epithelial cells of several glands but its role in lacrimal gland duct cells is largely unknown.

NKCC1 activity was measured using ammonium pulse technique and microfluorophotometry. NKCC1 can be characterized functionally during NH4+ pulse as bumetanide-sensitive, Na^+- and K^+-dependent $NH4^+$entry into the cells. In the first series of experiments we tested the hypothesis that NKCC1 can transport $NH4^+$ instead of K^+ with a resultant change in intracellular pH. Ammonium-induced acidification was reduced in the presence of high K^+ concentration in the superfusate, indicating competition between K^+ and NH_4^+ (Figure 6, panel A). To determine whether NH_4^+ transport in duct cells occurs via Na^+-dependent pathway, the NH_4^+ pulse was repeated with tetramethylammonium-chloride, instead of NaCl in the superfusate. Ammonium-induced cell acidification was reduced significantly in the absence of Na^+ (Figure 6 panel B). To test the existence of a bumetanide-sensitive basolateral transport system (indicative for the presence of NKCC1), standard NH_4^+ pulse was administered in the presence and absence of bumetanide. In the presence of bumetanide (100 μM), NH_4^+-induced cell acidification was significantly reduced in duct cells (Figure 6, panel C). Overall, these results confirmed the existence of a Na^+-dependent, bumetanide-sensitive pathway in the duct cells where K^+ transport is in competition with NH_4^+ suggesting the functional involvement of NKCC1.

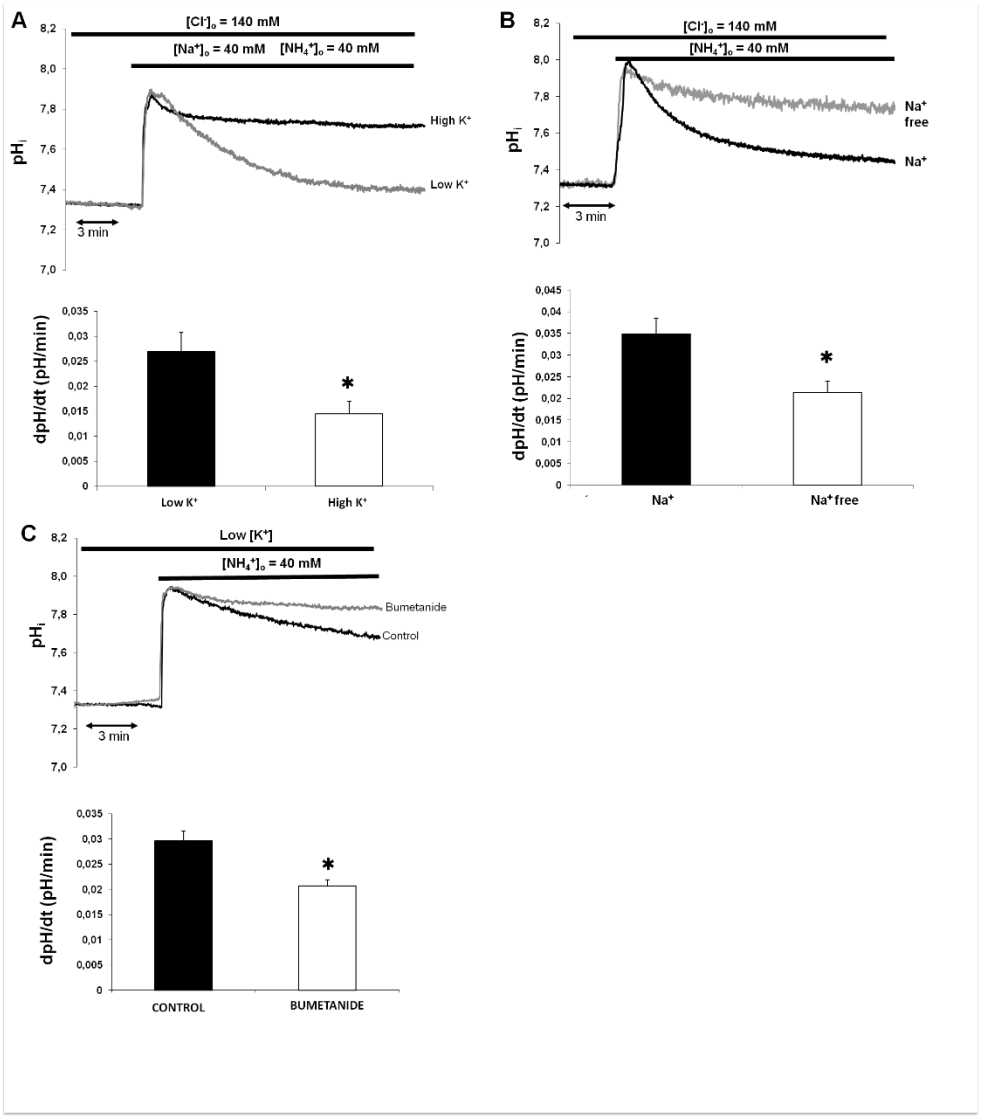

Figure 6. Functional involvement of NKCC1 in isolated rabbit lacrimal gland ducts. **A)** NH_4^- induced acidification during ammonium pulse is reduced by high K^+ content of the medium, indicating competition between NH_4^+ and K^+ transport. **B)** NH_4^+ transport process is Na^+ dependent. **C)** NH_4^+ transport is decreased by bumetanide. For each panel (A, B, C): top: representative curves of the experiments; bottom: initial rate of recovery from alkalosis (dpH/dt) over the first 60 seconds. Data was obtained from six ducts isolated from three different animals in each series. *: $p<0.05$.

Role of elevated cytosolic cAMP level (forskolin, cell permeable cAMP analoque 8-bromo cAMP), cholinergic stimulation (carbachol, Ca^{2+} ionophore A23187, phorbol 12-myristate 13-acetate), vasoactive intestinal peptide and various intracellular environmental factors (hyperosmolarity, low intracellular Cl^-) in the modification of NKCC1 activity of rabbit lacrimal gland ducts were investigated. Forskolin stimulation (i.e. elevation of cytosolic cAMP level) resulted in a marked increase of NKCC1 activity in isolated lacrimal gland duct segments. Similarly, cell permeable cAMP analogue 8-bromo cAMP also resulted in a significant elevation of NKCC1 activity. These results are consistent with our previous findings where the potent fluid secretory effect of forskolin could be completely blocked by the NKCC1 inhibitor bumetanide and suggest a decisive role of cAMP-dependent mechanisms and NKCC1 in ductal

fluid secretion. We investigated the role of parasympathetic pathway in the activation of NKCC1. We could not demonstrate notable effect of cholinergic agonist carbachol in the activation of NKCC1. Elevation of cytosolic Ca^{2+} level with Ca^{2+} ionophore did not cause activation of NKCC1 in our experiments. In contrast, direct stimulation of PKC with its potent activator PMA resulted in a significant increase of NKCC1 activity, even though the rate of activation of the cotransporter was very weak (Figure 7).

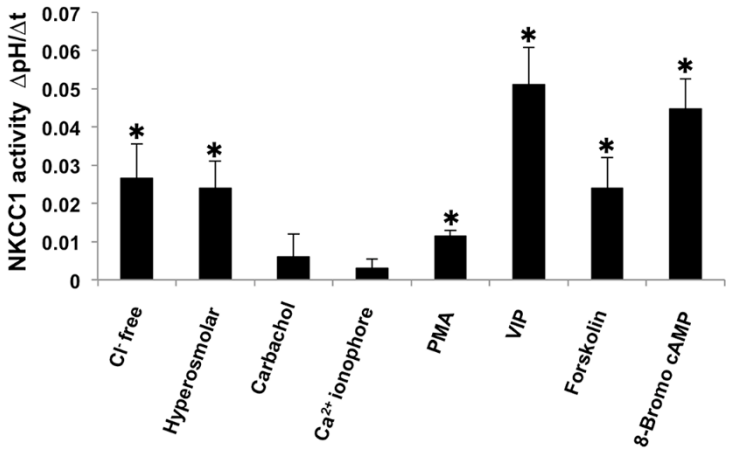

Figure 7. NKCC1 activity evoked by low intracellular Cl^- level, hyperosmolar environment and various secretagogues. NKCC1 activity was calculated from the rates of recovery from alkalosis over the first 60 seconds (dpH/dt) during ammonium pulse. *: $p<0.05$.

4) Investigation of the effect of vasoactive intestinal peptide (VIP) on lacrimal gland ductal fluid secretion

This study was performed in WT and CFTR KO mice. Immunofluorescence proved the localization of VPAC1 and VPAC2 receptors in mouse lacrimal gland tissue. Presence of VPAC1 was dominant in the duct cells as demonstrated in Figure 8. The receptor was localized on the basolateral surface of duct cells. Immunofluorescence staining of VPAC1 receptors revealed a mosaic pattern among different duct segments in the expression of the receptor proteins. Intensity of fluorescence varied excessively in the investigated duct segments from intense immunofluorescence to the lack of staining. No difference was detected in the immunoreactivity between lacrimal gland tissues from 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 9). Intensity of immunofluorescence staining was similar in both WT and CFTR KO lacrimal glands.

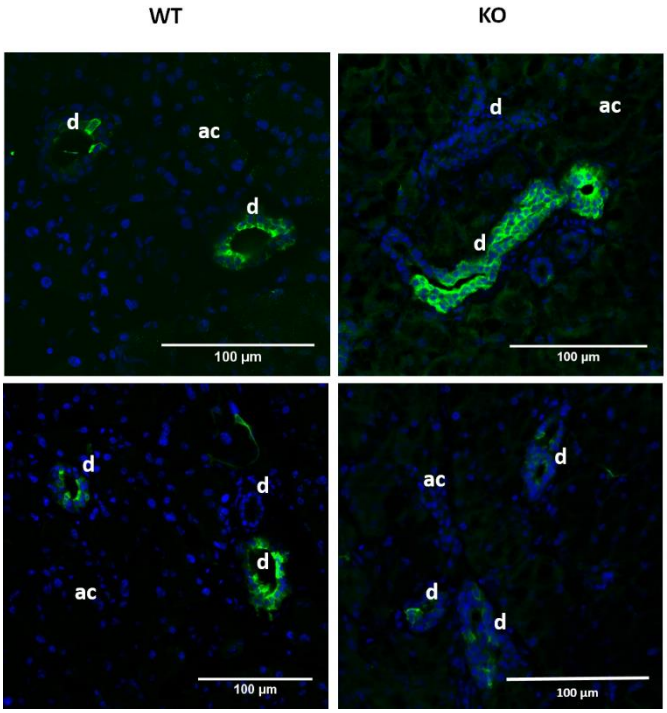

Figure 8. Immunfluorescence staining of VPAC1 receptors in lacrimal gland tissues of WT and CFTR KO mice. VPAC1 staining was more intense in ducts (d) than acinar (ac) cells. A mosaic pattern was observed in the expression of the receptor proteins in different ducts. Intensity of fluorescence varied excessively in the investigated duct segments from the intense immunofluorescence to the lack of staining. There were no significant differences between WT and CFTR KO samples. Hoechst was used to stain nuclei as blue.

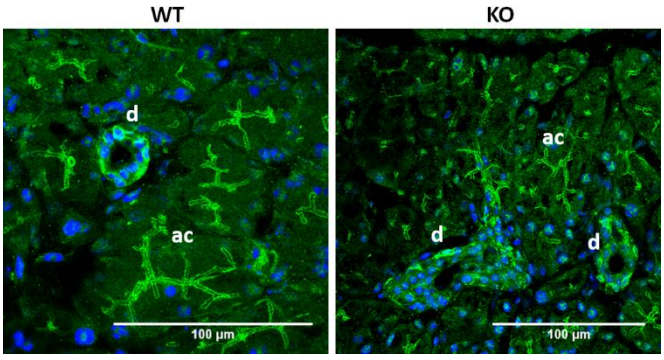

Figure 9. Immunfluorescence staining of VPAC2 receptors in lacrimal gland tissues of WT and CFTR mice. VPAC2 was observed in the basolateral surface of both duct (d) and acinar (ac) cells. There were no significant differences between WT and CFTR KO samples. Hoechst was used to stain nuclei as blue.

VIP stimulation resulted in a strong and continuous fluid secretory response in isolated WT mouse lacrimal gland duct segments. In contrast, CFTR KO ducts exhibited only a very weak pulse-like secretion in the first 5 minutes of stimulation, followed by a plateau phase (Figure 10).

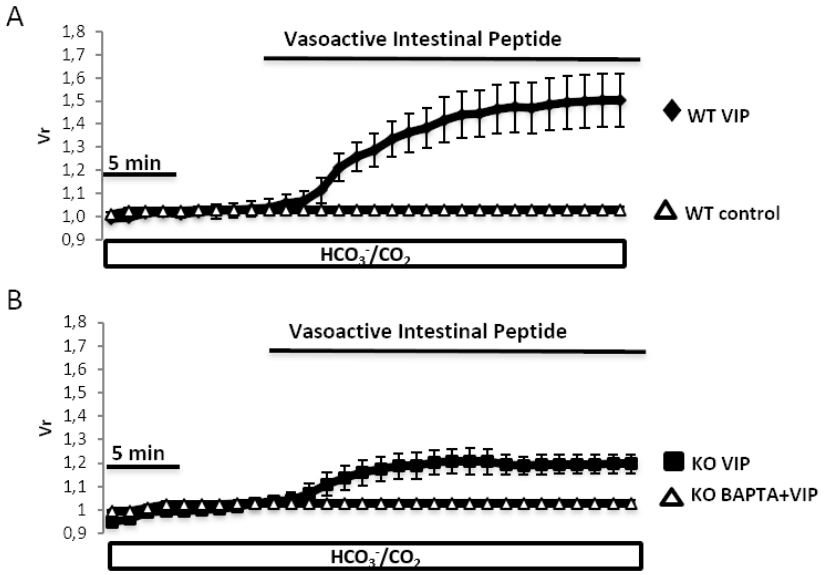

Figure 10. Effect of VIP stimulation on ductal fluid secretion in isolated lacrimal gland ducts from WT and CFTR KO mice. (A) WT ducts were exposed either to 100 nM VIP (filled rhombus) or to no agonist (empty triangle). (B) CFTR KO ducts were exposed either to 100 nM VIP (filled square) or to 100 nM VIP following 10 μM BAPTA-AM pretreatment (empty triangle). Changes in relative luminal volume (Vr) are shown. Data were obtained from 6 to 8 ducts isolated from 3 different animals in each series and are presented as means ±SEM.

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. Moreover, VIP stimulation resulted in a small, but statistically significant increase in cytosolic Ca^{2+} level both in WT and in CFTR KO ducts. 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. Secretory response given to VIP-stimulation was not detected following BAPTA-AM pre-incubation (Figure 10), and the secretory rates were similar to control values (ie. no secretion was detected either in the BAPTA-AM+VIP or in the control ducts). Our results reveal new insight into the role of VIP in lacrimal gland function by demonstrating VIP-induced fluid secretion of mouse lacrimal gland duct segments. Role of CFTR in the secretory process found to be decisive as only small VIP-induced fluid secretion was detected in the absence of CFTR protein. The weak fluid secretory response evoked by VIP in CFTR KO ducts may reflect the small elevation of cytosolic Ca^{2+} level observed.

5) Role of adrenergic agonists in the regulation of lacrimal gland ductal fluid secretion

Isolated lacrimal gland ducts originated from WT mouse 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 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. The most effective concentration of norepinephrine proved to be 10 μM (Figure 11). 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. To ensure the blockade of β-adrenergic receptors, phenylephrine was administered in the presence of β-adrenergic antagonist propranolol. Phenylephrine stimulation caused a rapid fluid secretory response in the isolated duct segments (Figure 11). The most effective concentration of phenylephrine found to be 10 μM. 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 vs. 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 to ensure the selective β-adrenergic stimulation. Isoproterenol failed to elicit any detectable secretory effect in all applied concentrations. Figure 11 exhibits secretory result of the highest isoproterenol concentration applied (200 μM).

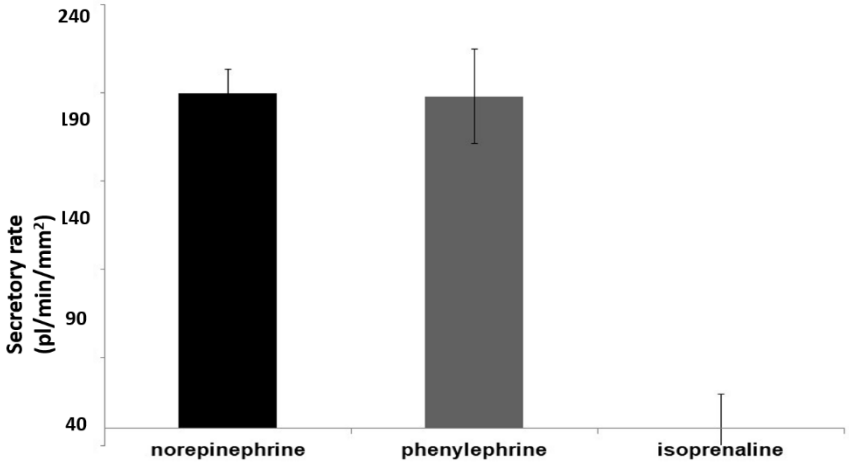

Figure 11. Effect of different adrenergic agonists on ductal fluid secretion in isolated lacrimal gland ducts. Isolated lacrimal gland ducts were stimulated with norepinephrine (10 μM), or with phenylephrine (10 μM) in the presence of propranolol (1 μM) or with isoproterenol (200 μM) in the presence of phentolamine (10 μM). Secretory response of ducts was measured with videomicroscopy. Changes in relative luminal volume (Vr) are shown. Data were obtained at least from 6 ducts isolated from 3 different animals in each series and are presented as means ±SEM.

Secretory response of isolated ducts suggested to be clearly due to the stimulation of α-adrenergic receptors in our experiments. Earlier studies demonstrated that α-adrenergic receptor subtype present in the acinar epithelial cells of lacrimal gland is the α_{1D}. Therefore, we investigated the effect of α_{1D}-adrenergic blockade to explore the subtype of the involved receptors in the isolated mouse lacrimal gland ducts. Duct segments were pre-incubated with selective α_{1D} receptor antagonist BMY-7378 for 30 minutes and then phenylephrine was added to the superfusate. BMY-7378 reduced phenylephrine-induced ductal fluid secretion in a dose-dependent manner proving the role of α_{1D}-adrenergic receptors in the observed secretory response (Figure 12).

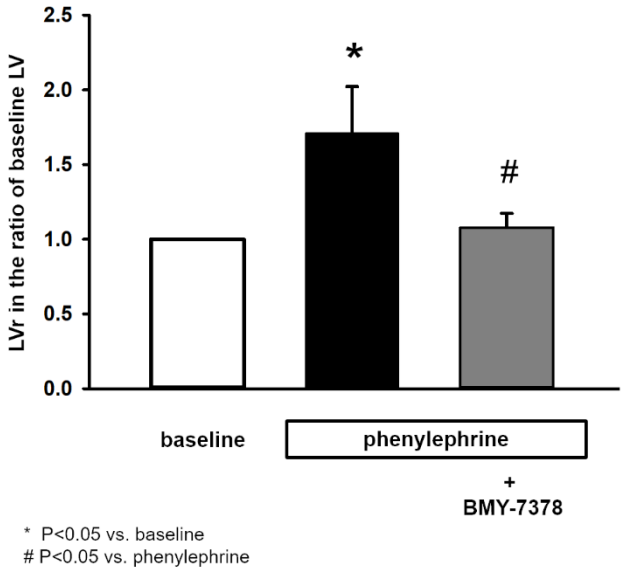

Figure 12. Effect of BMY-7378 pretreatment on phenylephrine induced secretory response of isolated lacrimal gland ducts. Isolated ducts were stimulated with phenylephrine (10 μM) either in the presence or in the absence of α_{1D}-receptor antagonist BMY-7378 (100 μM). Secretory response of ducts was measured with videomicroscopy. Changes in relative luminal volume (LVr) are shown. Data were obtained at least from 6 ducts isolated from 3 different animals in each series and are presented as means ±SEM.

The mechanisms underlying α_{1D}-adrenergic receptor stimulation was also investigated in our studies. Both eNOS inhibitor L-NAME and guanylyl cyclase inhibitor ODQ reduced, but not completely abolished the phenylephrine-induced fluid secretion of isolated LG ducts. In contrast, a complete inhibition of phenylephrine-induced ductal fluid secretion was observed following co-administration of either L-NAME or ODQ with Ca^{2+}-chelator BAPTA-AM.

Our data suggest the direct role of α-adrenergic stimulation in lacrimal gland ductal fluid secretion. Lack of isoproterenol-induced fluid secretory response suggests the absence of β-receptor mediated pathway in mouse lacrimal gland ducts. The similar secretory effects of norepinephrine and phenylephrine suggest that α-adrenergic stimulation is the determining adrenergic pathway 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.

Outcomes of the project:

Published full papers:

Vizvári E, Katona M, Orvos P, Berczeli O, Facskó A, Rárosi F, Venglovecz V, Rakonczay Z Jr, 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 Jul 1;57(8):3828-35.
[D1]

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 Jan 1;59(1):54-62.
[D1]

Tóth-Molnár E, Ding C:
New insight into lacrimal gland function: Role of the duct epithelium in tear secretion.
The Ocular Surface 2020 Jul 21;18(4):595-603. Review.
[D1]

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.***
Accepted for publication in **Molecular Vision**
[Q2]

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
Under minor revision in **Invest Ophthalmol Vis Sci.**

PhD Theses:

Vizvari E:
Role of transmembrane chloride transporters in the fluid secretion of lacrimal gland duct cells. Date of thesis defense: October 16, 2017

Berczeli O:
The role of parasympathetic regulation in the fluid secretion of lacrimal gland ducts in mice
Date of thesis defense: June 26, 2020

One PhD thesis is under preparation (Szarka D.). Date of defense is probable in Q1, 2021.