Physiology and Pharmacology

Characterization of Na⁺-K⁺-2Cl⁻ Cotransporter Activity in Rabbit Lacrimal Gland Duct Cells

Eszter Vizvári,1 Máthé Katona,2 Péter Orvos,3 Orsolya Berczeli,1 Andrea Facskó,1 Ferenc Rárosi,4 Viktória Venglovecz,5 Zoltán Rakonczay Jr,2,5 Péter Hegyi,2 Chuanqing Ding,6 and Edit Tóth-Molnár1,3

1Department of Ophthalmology, University of Szeged, Szeged, Hungary
21st Department of Internal Medicine, University of Szeged, Szeged, Hungary
3Department of Pharmacology and Pharmacotherapy, University of Szeged, Szeged, Hungary
4Department of Medical Physics and Informatics, University of Szeged, Szeged, Hungary
5Department of Pathophysiology, University of Szeged, Szeged, Hungary
6Pharmacology & Pharmaceutical Sciences, Ophthalmology, University of Southern California, Los Angeles, California, United States

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

OBJECTIVE. We recently reported that isolated duct segments from rabbit lacrimal gland (LG) were able to secrete fluid in response to secretagogues, which were blocked completely by bumetanide. This suggests the functional involvement of Na⁺-K⁺-2Cl⁻ cotransporter (NKCC1) in ductal fluid secretion. Therefore, the aim of this study was to investigate the activity profile of NKCC1 in isolated rabbit LG duct segments.

METHODS. Interlobular ducts were isolated from fresh rabbit LG tissue. Microfluorometry with the ammonium (NH₄⁺)-pulse technique was used to elicit pH changes in duct cells, and the rate of bumetanide-sensitive cytosolic acidification after addition of NH₄⁺ was used to quantify the activity of NKCC1.

RESULTS. While basal activity of NKCC1 was undetectable, low cytosolic chloride (Cl⁻) level and hyperosmotic challenge (390 mOsm) were able to increase the activity of NKCC1. Carbacol (100 μM) had no significant effect on NKCC1 activity. Elevation of cytosolic calcium (Ca²⁺) level with Ca²⁺-ionophore (A 23187, 1 μM) did not cause any alteration in the activity of the cotransporter while direct activation of protein kinase C (phorbol myristate acetate, 100 nM) increased its activity slightly but in a significant manner. Addition of either forskolin (10 μM), cell-permeable cAMP analogue (8-bromo cAMP, 100 μM) or vasoactive intestinal peptide (200 nM) resulted in a significant increase in the activity of NKCC1.

CONCLUSIONS. These results highlight the functional involvement of NKCC1 in LG duct secretion. These findings may facilitate our understanding of LG function and may contribute to the development of targeted pharmacologic interventions in case of dry eye disease.

Keywords: lacrimal gland, lacrimal gland, duct epithelium, duct epithelium, NKCC1, NKCC1

Purpose...
acinar and duct cells of rat, mice, and rabbit. Walcott et al. demonstrated the important role of NKCC1 in fluid secretion by using the whole LG in an in situ experimental setup they developed. However, until now there is no direct evidence regarding the functional role of NKCC1 in isolated LG duct epithelium. Therefore, we aimed to investigate the activity of NKCC1 in isolated rabbit LG duct segments.

Part of the results in this study have been reported in abstract form previously (Tóth-Molnár E, et al. IOVS 2015;56:ARVO E-Abstract 2482).

Methods

Animals

Adult male New Zealand white rabbits weighing 2 to 2.5 kg (Devai Farm, Kondoros, Hungary) were used throughout the studies. The animals were narcotized with a mixture of ketamine (10 mg/kg) and xylazine (3 mg/kg) and were euthanized with an overdose of pentobarbital (80 mg/kg). All experiments were conducted in compliance with the ARVO Statement 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.

Solutions and Chemicals for Isolation, Culture, and Perfusion of LG Ducts

Isolation solution contained Dulbecco’s modified eagle medium (DMEM) supplemented with 100 U/mL collagenase (Worthington, Lakewood, NJ, USA) and 1 mg/mL BSA. Storage solution contained McCoy’s 5A tissue culture medium, 10% (vol/vol) fetal calf serum (FCS), and 2 mM glutamine. Media supplements contained McCoy’s 5A tissue culture medium, 10% (vol/vol) fetal calf serum (FCS), and 2 mM glutamine. Media supplements (DMEM, McCoy, FCS, glutamine, and BSA) were purchased from Sigma-Aldrich (Budapest, Hungary). The composition of solutions used in our studies are summarized in the Table.

Carbachol (carbamoylcholine chloride), forskolin, bumetanide, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, 8-bromoadenosine-3′-5′-cyclic monophosphate (8-bromo cAMP), and vasoactive intestinal peptide (VIP) were purchased from Sigma-Aldrich. 2.7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) was purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

Isolation and Culture of LG Duct Segments

Rabbit LG interlobular ducts were isolated as described previously by our laboratory. In brief, LG was dissected and micro-dissected under a stereomicroscope and then transferred to the culture solution in a Petri dish. Ducts were bathed in standard HEPES solution at 37°C and loaded with the pH-sensitive fluorescent dye BCECF-AM (2 μM) for 25 minutes. Thereafter, the ducts were superfused continuously with solutions at a rate of 4 to 5 mL/min. Intracellular pH was measured using an imaging system (Cell; Olympus; Olympus Ltd). Four to six regions of interest (ROI) of 5 to 10 cells each in an intact duct were excited at 490 and 440 nm, respectively, and the 490/440 fluorescence emission ratio then was measured at 535 nm. One intracellular pH measurement per second was recorded.

Intracellular pH Measurement

After overnight culture, LG duct segments were carefully transferred to a coverslip (24 mm) pretreated with diluted (dilution ratio 1:9) poly-l-lysine (Sigma-Aldrich). The coverslip formed the base of a perfusion chamber mounted on an inverted microscope (Olympus; Olympus Ltd, Budapest, Hungary). Ducts were bathed in standard HEPES solution at 37°C and loaded with the pH-sensitive fluorescent dye BCECF-AM (2 μM) for 25 minutes. Thereafter, the ducts were superfused continuously with solutions at a rate of 4 to 5 mL/min. Intracellular pH was measured using an imaging system (Cell; Olympus; Olympus Ltd). Four to six regions of interest (ROI) of 5 to 10 cells each in an intact duct were excited at 490 and 440 nm, respectively, and the 490/440 fluorescence emission ratio then was measured at 535 nm. One intracellular pH measurement per second was recorded.

Measurement of NKCC1 Activity

Ammonium (NH4+) pulse technique was used to measure the activity rate of NKCC1. It was determined by the rate of intracellular acidification caused by NH4+ entry into the cells via this transport mechanism on abrupt application of NH4Cl as described by Shumaker et al. and Heitzmann et al. The theoretical background of this technique is the competition between NH4+ and K+ uptake as NKCC1 can accept NH4+ at its K+ binding site. Therefore, NKCC1 activity was determined by the rate of NH4+ uptake (activity rate of NKCC1). It was determined by the rate of change in intracellular pH. The addition of NH4Cl resulted in a four-phase curve representing the four-phase alterations in cytosolic pH: (1) rapid initial alkalization caused by NH3 entry into the cell, followed by (2) a slower decline in pH representing NH4+ uptake of the cell via NKCC1 (among other potentially contributing channels), (3) a rapid acidification after NH4Cl administration, and finally (4) the last phase represents the recovery of pH determined by hydrogen (H+) extrusion and bicarbonate (HCO3−) import. The kinetics of the second phase acidification is of particular interest with respect to NKCC1 activity, as this phase is associated with influx of NH4+, a substrate for NKCC1. Therefore, NKCC1 activity was determined as bumetanide-sensitive part of the second phase acidification representing bumetanide-dependent NH4+ entry into the cell. The slope of the second phase acidification was characterized by calculating the initial rates of recovery from alkalosis (dP/dt) over the first 60 seconds.

Statistics

A mixed ANOVA model was used for statistics, by using SPSS 22 software (IBM, Armonk, NY, USA). The effect of “bumetanide” was taken into account as a fixed effect. The effect of the individual “duct” and the “duct and effect of bumetanide” interaction (we assumed that the value of the effect of bumetanide depends on the individual duct) were taken into account as random effects in the model. P < 0.05 was considered as significant.

Results

Functional Involvement of NKCC1 in LG Duct Cells

In general, 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 superfuse (solutions A, C, and D, Table), indicating competition between K+ and NH4+ (low K+, 0.027 ± 0.002 pH unit/60 seconds; high K+, 0.014 ± 0.002 pH unit/60 seconds; P = 0.008, Fig. 1A).
Activation of NKCC1 by Low Cytosolic Cl−

To test the existence of a bumetanide-sensitive basolateral transport system (indicative for the presence of NKCC1), standard NH₄⁺ pulse was administered in the presence and absence of bumetanide (solutions A and B, Table). In the presence of bumetanide (100 μM), NH₄⁺-induced cell acidification was significantly reduced in duct cells (control, 0.029 ± 0.002 pH unit/60 seconds; bumetanide, 0.018 ± 0.002 pH unit/60 seconds; P = 0.004; Fig. 1C). Overall, these results confirmed the existence of a Na+-dependent, bumetanide-sensitive pathway in the duct cells where K⁺ transport is in competition with NH₄⁺ suggesting the functional involvement of NKCC1.

Influence of Repeated NH₄⁺ Pulse on the Slope of Second Phase Acidification

To determine whether repeated ammonium pulse administration itself could influence the slope of the second phase, three consecutive pulses were added to the same duct segment (solutions G and H, Table). The slope of the second phase was stable during repeated pulses; thus, the “fatigue” effect of the repeated base administration should not be taken into consideration during calculation of NKCC1 activity (pulse 1, 0.08 ± 0.006 pH unit/60 seconds; pulse 2, 0.08 ± 0.005 pH unit/60 seconds; pulse 3, 0.078 ± 0.006 pH unit/60 seconds).

Basal Activity of NKCC1

To determine whether basal activity of NKCC1 affects the measurements, the slope of second phase acidification was determined and compared during NH₄⁺ pulse in the presence and absence of bumetanide (solutions G and H, Table). Basal activity of NKCC1 was negligible and statistically not significant (0.009 ± 0.006 pH unit/60 seconds; P = 0.520).

Activation of NKCC1 by Low Cytosolic Cl−

Isolated LG duct segments were preincubated in Cl⁻-free solution for 20 minutes, then NH₄⁺ pulse was performed in the presence and absence of bumetanide in these experiments (solutions I and J, Table). The results are summarized in Figure 2. The bumetanide-inhibited component of the second-phase pH alteration during ammonium pulse represents the activation of NKCC1 by low cytosolic Cl−. Low cytosolic Cl− increased the activity of NKCC1, the increase was statistically significant (0.026 ± 0.009 pH unit/60 seconds; P = 0.025).

Activation of NKCC1 by Hyperosmolarity

To investigate the role of hyperosmotic environment in the activation of NKCC1, ducts were preincubated with bath solution of 390 mOsm/L in the absence and presence of bumetanide (solution G + 100 mM mannitol and solution H, Table). Hyperosmotic challenge, that is, elevation of bath osmolarity from 290 to 390 mOsm increased the activity of NKCC1 significantly (0.024 ± 0.007 pH unit/60 seconds; P = 0.025) indicating that hyperosmolarity has an important role in the activation of NKCC1 (Fig. 3).

Effects of Carbachol, PMA, and Ca²⁺ Ionophore A23187 in the Activation of NKCC1

Acetylcholine analogue carbachol was used to investigate the effect of cholinergic agonists in the activation of NKCC1. Isolated ducts were superfused with HEPES-Tris-phosphate medium, and NH₄⁺ pulse was administered in the absence and presence of bumetanide after preincubation with carbachol (100 μM, 5 minutes, solutions G and H, Table). Results are summarized in Figure 4A. The slope of second-phase acidification was increased compared to the control as a result of cholinergic stimulation. Bumetanide treatment (100 μM) did not change the slope of second phase acidification during NH₄⁺ pulse, indicating that carbachol had no significant effect on NKCC1 activity (0.006 ± 0.006 pH unit/60 seconds; P = 0.388).

To further elucidate the role of cholinergic cellular signaling pathways in the activation of NKCC1 in rabbit LG ducts, the effects of Ca²⁺ ionophore A23187 and protein kinase C (PKC) activator PMA was investigated. Preincubation with Ca²⁺ ionophore A23187 (5 min, 1 μM) was followed by NH₄⁺ pulse administration with and without bumetanide in the superfuse. Calcium ionophore A23187 did not result in activation of the cotransporter (0.002 ± 0.002 pH unit/60 seconds; P = 0.226, Fig. 4B). In the next series of experiments, effect of PMA was tested. Ammonium pulse was administered to the ducts in the absence and presence of bumetanide after preincubation with PMA (3 minutes, 100 nM, solutions G and H, Table).
Figure 1. Functional involvement of NKCC1 in isolated rabbit LG ducts. (A) Ammonium-induced acidification during NH_4^+ pulse is reduced by high K^+ content of the medium, indicating competition between NH_4^+ and K^+ transport. (B) Ammonium transport process is Na^+ dependent. (C) Ammonium transport is decreased by bumetanide. (A-C) Top: Representative curves of the experiments. Bottom: Initial rate of recovery from alkalosis (dpH/dt) over the first 60 seconds. Data were obtained from six ducts isolated from three different animals in each series. *P < 0.05.

Bumetanide treatment slightly but significantly reduced the slope of second phase acidification (0.011 ± 0.001 pH unit/60 seconds; P = 0.0007, Fig. 4C).

Activation of NKCC1 by VIP
Parasympathetic nerves release VIP in addition to the cholinergic agonist acetylcholine; thus, in the next series of experiments we investigated the effect of VIP in the activation of NKCC1. Isolated ducts were superfused with HEPES-Tris-phosphate medium and NH_4^+ pulse then was administered in the absence and presence of bumetanide after preincubation with VIP (5 minutes, 200 nM, solutions G and H, Table). The results are summarized in Figure 5. Vasoactive intestinal peptide treatment increased the rate of second phase acidification, which was reduced by bumetanide (100 μM).
The bumetanide-sensitive part of the second phase acidification was 0.051 ± 0.009 pH unit/60 seconds (P = 0.033) indicating the VIP-induced marked increase in NKCC1 activity.

**Activation of NKCC1 by Forskolin and Cell Permeable cAMP Analogue 8-Bromo cAMP**

Next, we studied the role of elevated intracellular cAMP level in the activation of NKCC1. Isolated ducts were superfused with HEPES-Tris-phosphate medium and NH₄⁺ pulse then was administered after a 5-minute incubation with forskolin (10 μM) in the absence and presence of bumetanide (solutions G and H, Table). The results are summarized in Figure 6A. Forskolin increased the rate of second phase acidification, which was reduced by bumetanide (100 μM). The bumetanide-sensitive part of the second phase acidification was 0.024 ± 0.008 pH unit/60 seconds (P = 0.045), indicating the forskolin-induced significant increase in NKCC1 activity.

To further verify the effect of elevated cytosolic cAMP level in the activation of NKCC1, cell permeable cAMP analogue was used. Ammonium pulse was administered in the absence and presence of bumetanide after preincubation with 8-bromo cAMP (100 μM, 5 minutes). The bumetanide-sensitive part of the second phase acidification was 0.044 ± 0.007 pH unit/60 seconds (P = 0.011), representing a statistically significant increase in NKCC1 activity caused by cell-permeable cAMP analogue 8-bromo cAMP (Fig. 6B).

**DISCUSSION**

Expression of NKCC1 in the duct cells of exocrine glands is remarkably species- and organ-specific. NKCC1 can be found in the basolateral membranes of rat and mouse pancreatic ducts, but not in the pancreatic and salivary gland duct cells of pig and guinea pig. Earlier we detected the expression of NKCC1 in the basolateral membranes of both acinar and duct cells from rabbit LG by immunofluorescence, with stronger staining observed in acinar cells. In the present study various factors that may influence the activity of NKCC1 in LG duct cells were investigated.

It is widely demonstrated that NKCC1 has a key role in volume regulation of cells, that is, cell shrinkage can be a potent signal of its activation. The precise molecular mechanism of how water transport is mediated by the cotransporter is unknown, but the consensus is that NKCC1 has a key role in this process. The ability of NKCC1 to couple water and ion transport may have a direct role in the secretory process in epithelial cells. Walcott et al. demonstrated the NKCC1-dependent manner of the regulatory volume increase in mouse LG acinar cells. In agreement with these previous findings hyperosmotic environment led to a marked increase of NKCC1 activity in rabbit LG duct cells.

We investigated the role of the parasympathetic pathway in the activation of NKCC1. We could not demonstrate a notable effect of the cholinergic agonist carbachol in the activation of NKCC1.
**Figure 4.** Effect of cholinergic signaling pathway on NKCC1 activity. Ducts were superfused either with (A) carbachol (carb, 100 μM), (B) Ca²⁺ ionophore A23187 (iono, 1 μM), or (C) PMA (100 nM) and ammonium pulse was administered in the absence and presence of bumetanide (bum, 100 μM). Initial uptake rate of NH₄⁺ was calculated and compared. Left: Representative curves of the experiments. Summary of data is shown at the right. Initial rate of recovery from alkalosis (dpH/dt) over the first 60 seconds is shown. Data were obtained from seven ducts isolated from three different animals in each group of experiments. *P < 0.05.

**Figure 5.** Effect of VIP on NKCC1 activity. Ducts were superfused with VIP (200 nM) and NH₄⁺ pulse was administered in the absence and presence of bumetanide (bum, 100 μM). Initial uptake rate of NH₄⁺ was calculated and compared. Left: Representative curves of the experiments. Summary of data is shown at the right. Initial rate of recovery from alkalosis (dpH/dt) over the first 60 seconds is shown. Data were obtained from five ducts isolated from three different animals. *P < 0.05.
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Figure 6. Effects of forskolin and cell permeable cAMP analogue 8-bromo cAMP on NKCC1 activity. Ducts were superfused either with forskolin (FSK, 10 μM, [A]) or 8-bromo cAMP (100 μM, [B]) and ammonium pulse was administered in the absence and presence of bumetanide (bum, 100 μM). Initial uptake rate of NH₄⁺ was calculated and compared. Left: Representative curves of the experiments. Summary of data is shown at the right. Initial rate of recovery from alkalosis (dPH/dt) over the first 60 seconds is shown. Data were obtained from five ducts isolated from three different animals in both groups of experiments. ∗P < 0.05.

NKCC1. This finding is in agreement with our previous results, that is, cholinergic stimulation of isolated rabbit LG duct segments resulted in a very weak fluid secretory response.11 To further characterize the role of the cholinergic cellular signaling pathways, potential effects of a Ca²⁺-ionophore (A23187) and PKC activator (PMA) were measured. Elevation of cytosolic Ca²⁺ level with Ca²⁺-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. This contradiction between carbachol effect (no activation of NKCC1) and PMA effect (activation of NKCC1) might be explained by the weaker extent of activation of PKC during cholinergic effect compared to the direct and robust activation of the enzyme by PMA. Lack of effect of carbachol on NKCC1 also might be explained by the rapid cholinergic-evoked internalization of the cotransporter.26 This later effect could be a possible explanation by which carbachol and forskolin influence the activity of the cotransporter in different ways.26,27 This theory is in agreement with our results, and explains, at least in part, the different fluid secretory patterns of NKCC1 in our experiments. In conclusion, our results demonstrated the functional presence of NKCC1 in rabbit LG duct cells, providing further support that this transporter can be the main route of basolateral Cl⁻ uptake. We found that low cytosolic Cl⁻ level caused a significant increase in the activation of NKCC1. Hyperosmolarity of bath media, which results in cell shrinkage, proved to be a potent activator of NKCC1. NKCC1 also could be activated by elevated cytosolic cAMP level, VIP treatment, and, in a considerably smaller extent, by direct activation of PKC (Fig. 7).

Besides acetylcholine, parasympathetic nerves also release VIP. Earlier reports demonstrated the presence of VIP receptors on acinar and duct cells of LG. Thus, we investigated the effects of VIP in the activation of NKCC1.28 This transmitter acts predominantly through elevation of cytosolic cAMP level, the minority of its action thought to be mediated by Ca²⁺ signaling. We could demonstrate a considerable increase of NKCC1 activity evoked by VIP stimulation. Further studies are needed to investigate the effect of VIP on ductal fluid secretion.

In our present study we found that forskolin stimulation (i.e., elevation of cytosolic cAMP level) resulted in a marked increase in NKCC1 activity in isolated LG 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 blocked completely by the NKCC1 inhibitor bumetanide and suggest a decisive role of cAMP-dependent mechanisms and NKCC1 in ductal fluid secretion.11

In conclusion, our results demonstrated the functional presence of NKCC1 in rabbit LG duct cells, providing further support that this transporter can be the main route of basolateral Cl⁻ uptake. We found that low cytosolic Cl⁻ level caused a significant increase in the activation of NKCC1. Hyperosmolarity of bath media, which results in cell shrinkage, proved to be a potent activator of NKCC1. NKCC1 also could be activated by elevated cytosolic cAMP level, VIP treatment, and, in a considerably smaller extent, by direct activation of PKC (Fig. 7).

Figure 7. Activity of NKCC1 evoked by low intracellular Cl⁻ level, hyperosmolar environment and various secretagogues. Activity of NKCC1 was calculated from the rates of recovery from alkalosis over the first 60 seconds (dPH/dt) during ammonium pulse. ∗P < 0.05.
More detailed understanding of LG function is essential to develop novel approaches in the treatment of dry eye disease, which is an increasing health care problem in the industrialized countries. Unfortunately, duct cells have been understudied for many years compared to acinar cells, although recent advances clearly indicated that these duct cells have critical and indispensable roles in LG production. Further studies certainly are needed to investigate the role of apical transport processes and the interaction between basolateral and apical transport mechanisms underlying ductal fluid and electrolyte secretion. These future results may further facilitate our understanding of lacrimal gland function and may contribute to the development of targeted pharmacological interventions in case of dry eye disease.

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