Fluid secretion and the \(Na^+\)-\(K^+\)-\(2Cl^-\) cotransporter in mouse exorbital lacrimal gland

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Submitted 28 October 2004; accepted in final form 21 May 2005

A major secretory product of the lacrimal gland is fluid that hydrates the ocular surface and transports tear-specific proteins produced in the lacrimal gland to the ocular surface (47). Fluid secretion in lacrimal glands is an osmotic process driven by ion movement through the apical membrane. Upon stimulation by muscarinic agonists, cytosolic \(Ca^{2+}\) ion concentration increases (43) causing apically located \(Cl^-\) channels to open (9, 22, 33, 41, 46). \(Cl^-\) ions move into the lumen of the acinus (41). The rise in \(Ca^{2+}\) also activates large \(K^+\) channels (BK) in both apical (48) and basolateral membranes causing an efflux of \(K^+\) out of the cell (8, 12, 28, 31, 38, 46). This efflux of \(K^+\) ions hyperpolarizes the membrane slightly (3–5 mV) moving it further below the \(Cl^-\) equilibrium potential (41). This allows both \(Cl^-\) and \(K^+\) ions to move down their electrochemical gradients into the lumen of the acinus and water follows by osmosis.

To maintain significant ion and water secretion, the intracellular concentrations of \(K^+\) and \(Cl^-\) must remain sufficiently high to drive the passive transport through the apical membrane. In other tissues that transport water using similar mechanisms (e.g., cornea, salivary gland), there are several cotransporter systems that serve to replenish the cells with \(Cl^-\) and \(K^+\). One system is the \(Cl^-/\text{HCO}_3^-\) exchanger acting in concert with the \(Na^+\)/\(H^+\) exchanger (24) and another is the \(Na^+\)-\(K^+\)-\(2Cl^-\) cotransporter (NKCC1) (10, 14, 15, 20). Although the NKCC1 has been identified cornea and salivary gland, where its functional significance is well established, there is only fragmentary evidence for its presence and role in fluid secretion in lacrimal gland. The mRNA for the transporter has been reported to be present in rabbit lacrimal glands (19) and application of furosemide, an inhibitor of NKCC1, reduced \(Cl^-\) ion flux (35) as well as fluid flow measured on the ocular surface in situ (2). There are no published data on mouse lacrimal glands, which are often used as model systems for dry-eye disease. Therefore, we have examined mouse exorbital lacrimal glands to determine if NKCC1 is present and to evaluate its role in fluid secretion. We show using immunocytochemical methods that antisera against NKCC1 bind to the plasma membranes of acinar and duct cells. Furthermore, we developed a new method for continuous topical drug application to lacrimal glands in situ that was used to demonstrate that furosemide treatment reduced fluid flow measured in the lacrimal duct. Finally, we demonstrate that furosemide blunts stimulus-induced shrinkage in lacrimal acinar cells in primary culture. Together, these data strongly suggest a major role for the NKCC1 transporter in fluid secretion by the mouse exorbital lacrimal gland.

**METHODS**

**Animals.** Experiments were conducted in C57 mice (Teconic). They were maintained in an approved animal facility, and all procedures followed an approved Institutional Animal Care and Use Committee protocol.

**Immunocytochemistry.** Glands were removed and fixed for 4 h at 4°C in 4% parafomaldehyde in 0.1 M phosphate buffer. The tissue was then placed in 30% sucrose in buffer at 4°C for at least 12 h and up to 5 days. The tissue was frozen in frozen tissue medium and then cut on a cryostat at 14 μm. Sections were collected on slides, dried and then stored at −20°C until staining. Staining was accomplished by rehydrating the slides in buffer (0.1 M phosphate buffer, pH 7.4, with 0.25% Triton X-100) for 30 min. The tissue was then treated with nonspecific goat serum (Cappel Labs) for 1 h at room temperature. The slides were then drained and exposed to either of three primary antibodies to NKCC1. One was supplied by Dr. Turner (30) and was used at 1:1,000 dilution. Another antiserum was provided by Dr. Turner (30) and was used at 1:1,000 dilution. Another antiserum was provided by Dr. Turner (30) and was used at 1:1,000 dilution. Another antiserum was provided by Dr. Turner (30) and was used at 1:1,000 dilution.

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Delpire (21) and was used at 1:200 dilution. Both antisera were made in rabbits. The third primary antiserum used was from Santa Cruz Biotechnology (Santa Cruz, CA), and was made from goat serum. It was used at a dilution of 1:100. After an overnight incubation at room temperature in a humidified chamber, the slides were drained and washed for 1 h in the buffer with Triton X-100. As controls, some slides were exposed to normal goat serum instead of the primary antiserum. All slides were then exposed to secondary goat anti-rabbit antisera coupled to FITC (Cappel) diluted 1:75 for 1 h at room temperature. The slides were then washed in buffer plus Triton X-100 for 1 to 2 h and were cover-slipped using Vectashield medium (Vector Laboratories). The sections were examined using a Zeiss epifluorescence microscope and photographed using a Sony DKC 5000 digital camera.

In vivo measurement of lacrimal secretion. Mice were anesthetized with Inactin/ketamine (110–130 mg/kg, Inactin, intraperitoneal; 100–120 mg/kg ketamine, intramuscular) and placed on a heated surgical table. Rectal temperature was maintained at ~37°C. A tracheal cannula was inserted to avoid aspiration of saliva. Catheters were inserted into the left jugular vein (for isotonic saline infusion of 0.5 ml/h, 100 g body wt−1 and additional anesthetic as needed), and into the right femoral artery for arterial pressure monitoring and blood sampling. Heparin was added to the fluid in the arterial line to prevent clotting.

The experimental procedure is illustrated in Fig. 1. The animal is placed on its side and the head is immobilized with nonpenetrating steel pins. The right lacrimal gland is exposed with a small incision along an axis defined by the outer junction of the eyelids and the ear. The duct and gland were then freed via blunt dissection under a stereo microscope. Care was taken not to injure nearby nerves and blood vessels. The thin connective tissue capsule enclosing the gland was then carefully opened and removed from the upper surface to maximize penetration of topically applied drugs and chemicals.

To examine the effect of transport inhibitors on lacrimal function, we developed a method to continuously expose the in situ lacrimal gland to drugs and secretagogues. The motivation was that muscarinic agonists, when administered intravenously, had cardiodepressive effects that were sufficient to reduce arterial pressure to very low levels (<50 mmHg). Continuous topical application was accomplished by superfusing the exposed lacrimal gland at ~0.5–1.0 ml/min with mock interstitial fluid [Kreb’s bicarbonate Ringer (KBR) solution: 105.1 mM NaCl, 16 mM sodium acetate, 0.6 mM Na2HPO4, 1.19 mM MgSO4, 4.84 mM KCl, 2.2 mM CaCl2, 5.55 mM glucose, 24.99 mM urea, 0.13 mM NaH2PO4, 5.0 mM HEPES, and 1% dialyzed BSA, pH adjusted to 7.4]. The temperature of the superfusate, measured at the end of the ~1.5 mm interior diameter superfusion cannula, was servocontrolled at 37°C with a temperature control system (TC-1 CellMicro Controls). The heating element was incorporated into the superfusion cannula. To limit the spread of the superfusate (which could interfere with the flow measurements), the gland was covered with two layers of a fine cellulose fiber mesh (Kimwipe) cut in the shape of the exposed gland but with a 2- to 3-mm × 3- to 4-mm tab that extended beyond the ventral edge of the gland. The first layer of fiber mesh layer was placed on the gland, and the superfusate cannula was positioned so that it was ~0.5 mm above the surface of the mesh at the dorsal aspect of the gland. The second layer of tissue was then placed so that it covered the tip of the superfusion cannula as well as the whole gland. Fluid was drained from the mesh with a rigid aspiration pipette positioned with a micromanipulator to apply suction to the tab of fiber mesh just below the gland. This arrangement resulted in a thin layer of superfusate flowing across the top of the gland, and the spread of the superfusate was prevented by surface tension and the capillarity of the fiber mesh. Drugs and chemicals could be added to the superfusate, and the system had a wash-in time of ~30 s.

The lacrimal duct was then sectioned as far distally as possible (~70–80% of the duct remains), and the severed end of the duct was placed into a constant bore glass microcapillary tube (Microcaps; Drummond Scientific) that had been fire polished briefly. By using microcaps of different volumes (1, 2, and 5 μl), both basal and stimulated flows could be measured. The inner diameter of the collection capillary must be sufficiently large to contain the duct without impeding flow; the total time of collection was limited by the volume of the collection tube. Fluid collection into the capillaries occurred spontaneously, and we measured the rate of fluid flow by monitoring the movement of the fluid meniscus with a charge-coupled device camera mounted on a stereomicroscope. Recordings of the images were obtained using a video printer or on a S-VHS videocassette recorder. Minute flows were measured on the recordings by determining the increase in length of the fluid column per minute and multiplying by the volume per unit length for the capillary tube.

After a 15-min recovery period with continuous superfusion, the duct was placed into the collection tube, and one of two protocols was used. In the first protocol, the superfusate was switched to a KBR solution containing 10 μM carbachol. This resulted in rapid increase in lacrimal secretion that declined to a slowly waning plateau (see Fig. 3). After 10 min, the perfusate was switched to KBR solution with 100 μM furosemide without carbachol. After an additional 10 min, the superfusate was switched to a KBR solution containing furosemide and 10 μM carbachol. The flow response was then monitored for 10 min. After the flow response was measured, the superfusate was switched to KBR solution for 20 min, after which a repeat measure of the flow response to carbachol was obtained. The magnitude of the early peak in flow was highly variable and was attenuated with repeated stimulation in an individual gland, whereas the magnitude of the plateau phase was more consistent (see Fig. 3). Hence, we computed the flow-time average during the last 3 min of the plateau for each response and compared the value with furosemide with the average of the pre- and postfurosemide responses.

In the second protocol, we compared the effect of furosemide exposure beginning at the start of the plateau phase with time controls from the same mouse. Five minutes after exposure to KBR with carbachol, the superfusate was switched to KBR with carbachol and furosemide for an additional 5 min. The change in flow between minute 5 and the average of the flow during minutes 7–10 was calculated and compared with time controls not exposed to furosemide.

Ion concentration in lacrimal fluid. Some tear fluid samples were sealed in the collection tubes with silicone clay (CristoSel) for determination of Na+ and K+ ion concentrations with ion-selective microelectrodes obtained from commercial sources (Diamond General). Aliquots (~500 nl) were obtained from the tear samples using an oil-filled micropipette and transferred into the lumen of a 2-μl microcapillary tube. The aliquots were placed within a column of mineral oil to prevent evaporation. The ion-selective electrode and an appropriate reference were then inserted into the lumen of the capillary tube into the sample. The resulting potential was measured with a Keithley

Fig. 1. Diagram showing the method used to measure lacrimal gland secretion rate in vivo. CCD, charge-coupled device.
electrometer. The measurement of sample potential was performed in duplicate, and each determination was bracketed by potential measurements in similar aliquots of standard solutions. Corrections for cross-selectivity between K⁺ and Na⁺ ions were computed using selectivity coefficients provided by the vendor. Cl⁻ ion concentrations were measured in nanoliter aliquots by performing electrochemical titration in a bath of water-equilibrated mineral oil (39).

Preparation of cultured acinar cells. Cells are isolated using a method modified from that of Hann et al. (16) The glands are removed, placed into a soybean trypsin inhibitor (STI), and cut into small pieces using two sharp, sterile scalpels. The pieces were then washed with Hanks’ balanced salt solution (11885-084; GIBCO, Grand Island, NY) and incubated at 37°C for 15 min. After being washed again with STI, the cells were incubated in a 37°C agitated mixture of collagenase, DNase, and hyaluronidase in Dulbecco’s modified Eagle’s medium for 25 min. The resulting mixture was centrifuged at 1,200 rpm for 5 min, and the pellet was resuspended in medium. The suspension was filtered through sterile mesh to remove the large fragments that remained, and the resulting cell suspension was centrifuged again. The pellet was resuspended in medium, and 0.5-ml aliquots were plated into small sterile dishes that had small coverslips on the bottom that were coated with Matrigel. The plates were kept in a tissue culture incubator overnight and used the next day.

Measurement of carbachol-induced acinar cell shrinkage. Stimulation of secretory epithelial cells resulted in cell shrinkage, owing to a net steady-state loss of intracellular solute during active fluid secretion (7). Harvested acinar cells were plated on Matrigel-coated glass coverslips during overnight incubation at 37°C. After being washed with mammalian Tyrode solution, coverslips were placed into a chamber on an inverted microscope and perfused with a warmed (37°C) isotonic (320 mosmol/l) solution (in mM: 125 NaCl, 20 NaHCO₃, 4 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, and 5 HEPES, adjusted to pH 7.4 after equilibration with 95% O₂-CO₂). After 30-min rest, control images of the cell were obtained during the course of 3 min via a videomicroscopic system through a ×40 lens objective. The perfusate was then switched to one of five test solutions (control Tyrode solution plus carbachol + 100 µM furosemide, Tyrode solution plus 80 mM mannitol, and Tyrode solution plus 80 mM mannitol plus 100 µM furosemide). Images of the cell were obtained every 30 s after application of the test solution for 10–20 min. Cell volumes (V) were estimated from the videotape images by outlining the cells and computing the enclosed area (A) using NIH Image software. Relative volume was then computed as follows:

\[ V(t)/V_0 = (A(t)/A_0)^{1/2} \]

Statistical analysis. Comparisons were performed with paired or two-sample t-tests as indicated. Comparisons of cell volume responses were performed using two-way ANOVA, followed by the Student-Newman-Keuls multiple-comparison test using SigmaStat software (Systat).

RESULTS

Immunocytochemistry. All three primary antisera against NKCC1 showed significant staining of the mouse lacrimal gland as shown in Figs. 2 and 3. There was staining of plasma membranes of both the acinar cells and the duct cells within the lobes of the gland. In acinar cells (Fig. 2), it was difficult to determine whether the staining included only the basolateral membrane or the entire plasma membrane, because the lumen was difficult to visualize in frozen sections. In occasional fortuitous sections, it was possible to observe that there was little staining of the apical membrane compared with the basolateral membranes (Fig. 2, inset). In duct cells (Fig. 3) in which there is good definition of the apical membrane, it was clear that the staining was exclusively on the basolateral membrane. The staining of duct cell membranes was more intense than that of the acinar cells. There was little evident intracellular staining of either duct or acinar cells.

In situ lacrimal gland secretion. Carbachol-stimulated tear or fluid flow from the lacrimal gland is shown in Fig. 4. A typical response was an initial maximal flow rate of ∼0.25–0.6
μL/min within the first 2 min. The flow subsided within a few minutes to a plateau, at which time the decline in flow was considerably slower. The peak was markedly attenuated upon repeated stimulation of the gland as shown in Fig. 4, which shows the time course of second stimulations in the same seven glands. These glands were allowed 20-min recovery time before repeat stimulation. In contrast, the magnitude of the plateau phase of the response was less variable upon repeat stimulation. Because of the variability of peak flow, we generally compared responses on the basis of the magnitude of the average plateau phase flow averaged during the last 3 min of carbachol stimulation.

Monovalent ion concentrations were measured in tear samples from four mice. The results (means ± SE) were 38 ± 5, 144 ± 5, and 149 ± 16 mM for K⁺, Na⁺, and Cl⁻, respectively. Because ion-selective microelectrodes provide a measure of ion activities, the actual cation concentrations in the samples may be somewhat higher. Nevertheless, the high K⁺ concentration is in agreement with previous measurements in rat lacrimal fluid (1), and the sum of the cation concentrations are consistent with the finding that lacrimal fluid is significantly hypertonic with respect to plasma.

Figure 5 shows the effect of furosemide on carbachol-stimulated lacrimal gland fluid secretion. The data show that 10-min preexposure to furosemide reduced fluid secretion (Fig. 5, inverted triangle) compared with the average of the bracketing preexposure and recovery responses (Fig. 5, closed circle) obtained in the same glands. The difference in flows (Fig.
by the cell. The resulting increased cellular solute caused the cells to swell when exposed to isotonic bathing fluid. In acinar cells, both the RVI and the post-RVI cell swelling were essentially abolished in the presence of furosemide (Fig. 6A, open circle) a finding consistent with the well-established

Cell volume studies. Although the results from the in vivo fluid secretion measurements are consistent with the concept that NKCC1 participates in acinar cell ion transport, it is difficult to know the extent of NKCC1 blockade when furosemide is applied topically to the intact gland in situ. To address this issue, we conducted similar experiments using isolated lacrimal cells in vitro under conditions in which exposure to furosemide was better controlled. Figure 6A shows that isolated acinar cells exhibited responses to hyperosmotic shock (Fig. 6A, closed circle), which are typical of many other cell types, including rat lacrimal gland cells (7, 37). The initial cell shrinkage was followed by a slower regulatory volume increase (RVI), which reflected the uptake of additional solute

Fig. 5. Graph showing the effects of furosemide on lacrimal gland fluid secretion rate. Pretreatment (●) with furosemide (10 μM) reduced the rate of fluid secretion induced by carbachol alone (○). The paired difference in flows (○) was significant for minutes 2-10 (P < 0.05, paired t-test; n = 7). In each gland, the control curve was the average of prefurosemide and recovery responses to carbachol.

Fig. 6. Summary of cell volume studies. A: response of primary culture acinar cells to hyperosmotic shock. The cells exhibited a typical regulatory volume increase (RVI) in response to the initial shrinkage. When an isotonic bath was restored, cell volume increased above baseline, reflecting net solute uptake during RVI. The RVI and the posthypotonic cell swelling were both blocked by furosemide (P < 0.05, two-sample t-test; n = 8 per group). B: carbachol-induced acinar cell shrinkage. The reduction in cell volume reflects the net solute loss that occurred during the transition to the secretory state. The response was blocked (○) by atropine (0.1 mM), which inhibited the muscarinic receptors. The extent of carbachol-induced cell shrinkage (●) was attenuated by exposure to furosemide (●) beginning 2 min before application of carbachol. P < 0.05, response different from time control and carbachol alone (two-way ANOVA, Student-Newman-Keuls test; n = 8 per group). C: effects of 20-min furosemide pretreatment (●) on cell volume. During pretreatment, cell volume slowly declined; the loss of cell volume was blocked by flufenamic acid (10 μM), a Cl− channel blocker (○). A further decrease in cell volume was observed when the cells were exposed to carbachol (n = 5).

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concept that the NKCC1 transporter mediates the volume regulation of the cell (7, 14).

Figure 6B demonstrates the effects of muscarinic receptor stimulation on acinar cell volume. Cells began to shrink immediately when carbachol (10 μM) was introduced (Fig. 6B, closed circle), and volume stabilized at ~90% of the initial value. The loss of volume reflected a net loss of intercellular solute as the cell switched from the resting to the stimulated state. Cell volume eventually stabilized when ion uptake rose to a level sufficient to balance the rate of ion secretion. This response was fully blocked by atropine (Fig. 6B, open circle), indicating that it was mediated by muscarinic receptors. When furosemide was added 2 min before the carbachol challenge (Fig. 6B, inverted triangle), the magnitude of the resultant cell shrinkage was reduced by ~50%.

The effects of 20-min preexposure to furosemide are shown in Fig. 6C. These studies were undertaken because the interval of furosemide pretreatment corresponded to the measurement of in vivo lacrimal flow as presented in Fig. 5. After a latent period of several minutes, cell volume began a slow decline of ~20% in volume for 20 min. Exposure to carbachol at that point elicited a further, more rapid decrease in cell volume. The slow loss of solute before carbachol exposure likely represented the leak conductance of unstimulated the acinar cells. The leak pathway required Cl− conductance, because the cell shrinkage was completely blocked by 0.1 mM flufenamic acid, an inhibitor of Cl− ion channels (Fig. 6C).

Previous patch-clamp measurements of membrane currents induced by carbachol in isolated cells showed that there were two dominant currents: an inward Cl− current and an outward K+ current (3, 11). Application of furosemide with carbachol did not change the magnitude of either of these currents (data not presented). Flufenamic acid exposure reduced the Cl− current but did not greatly affect the K+ current (11).

DISCUSSION

The results of this investigation provide evidence that NKCC1 plays a significant role in fluid secretion by the mouse lacrimal gland. The immunocytochemistry showed clear membrane staining of both acinar and duct cells with antisera to the NKCC1 transporter. Because of the small area of the acinar apical membrane and the use of frozen sections, it was difficult to determine whether the staining of acinar membranes was restricted to the basolateral membranes. In some images (see Fig. 2, inset), it did seem as if there was little staining of the apical membrane. However, it was clear in the case of duct cells (Fig. 3) in which the duct had a clear lumen and the cells had larger apical membranes that the staining was observed only on the basolateral membranes with no staining of the apical membranes. Both rabbit antisera used in this study have been used previously in salivary gland tissues as well as in cornea (20, 30). In cornea, the staining was found on the basolateral membrane and not on the apical membrane (20). The salivary gland showed staining of the acinar cell plasma membranes similar to that observed in the present study in the lacrimal gland acinar cells (30). In salivary glands, however, the duct cells failed to show any staining (27), in contrast to our observations in the lacrimal gland, where staining was more intense in these cells than in the acinar cells. However, acinar cells are larger than duct cells, and it has been estimated that they comprise ~80% of the cells in the gland. Therefore, although the staining of acinar cell membranes was less intense, suggesting a lower density of NKCC1 protein, they are still likely to express quantities of NKCC1 sufficient to play a significant role in fluid secretion in the gland.

In salivary glands, the duct cells are known to play an active role in the exchange of Na+ for K+ in the salivary fluid (49). However, the localization of the NKCC1 on the basolateral membranes of lacrimal duct cells, together with the measurement of the composition of fluid collected from the distal lacrimal duct, suggests that lacrimal duct cells may be fundamentally different from salivary gland duct cells. Unlike salivary secretions that are hypotonic, high in Na+ and Cl−, and low in K+ (44), the lacrimal gland fluid was hypertonic and high in both Cl− and K+. The localization of NKCC1 on the basolateral membrane rather than on the apical membrane of the duct cells suggests K+ and Cl− secretion rather than absorption (14).

The high density of staining observed on the duct cells certainly suggests active ion transport. However, the question of the mechanisms of ductal cell transport and whether this is associated with substantial water transport is unclear. The basis for this assertion is data provided in the study by Alexander et al. (1) showing that the composition and the degree of hypertonicity of lacrimal fluid collected from the terminal duct is remarkably independent of flow rate. At very low flows, the concentration of K+ and Na+ was higher, but the degree of hypertonicity did not appear to change. If the ductal epithelium were sufficiently water permeable to permit osmotic water transport secondary to ductal cell ion transport, one would clearly expect that the hypertonicity of the lacrimal fluid would be dissipated at low flow rates. Because salivary and pancreatic ducts and the distal renal tubule all show a strong dependency of ion composition on fluid flow, the transport process in the ductal system of the lacrimal gland appears to be unique and worthy of further study. In this regard, the delivery of hyper tonic fluid to the ocular surface may be of physiological importance because it would promote osmotic water transport into the tear film from the cornea and conjunctiva.

We evaluated the physiological role of the NKCC1 transporter with two complementary techniques: a new method for the measurement of lacrimal gland secretion in vivo and evaluation of secretagogue-induced changes in isolated acinar cell volume. The new method provides continuous topical exposure to test substances without intravenous administration; in our experiments, the use of intravenous carbachol often resulted in severe hypotension in anesthetized mice. The method also permits measurement of the temporal pattern of secretion and the collection of samples of the fluid secreted by the lacrimal gland. The primary disadvantages of the method are that the extent of penetration of test substances is difficult to quantify and that the access of hydrophobic transport inhibitors to the apical membrane may be limited in an intact gland. These limitations are obviated in studies of isolated acinar cells, in which consistent drug exposure to both basolateral and apical membranes can be achieved. However, isolated cells are subjected to some degree of trauma, and cell volume changes are only an indirect indicator of ion efflux across the apical membrane.

The data obtained from intact, in situ lacrimal glands reveal an interesting temporal pattern in fluid secretion after stimula-
tion. In the majority of glands, the initial stimulation was associated with a prominent early peak flow of highly variable magnitude that quickly subsided to a lower, more stable plateau. Flow in the plateau phase of the response was relatively stable for up to 20 min in most glands; in some glands, however, the flow waned after 10 min of stimulation. The mechanism responsible for the flow peak is unclear. The fact that the peak was attenuated on subsequent stimulations to a greater degree than the magnitude of the flow in the plateau phase suggests a minor role for receptor desensitization. It is unlikely that the reduced flow as a result of the second and subsequent stimuli were due to cell death, because we have been able to collect fluid for several hours and the volume of fluid collected per unit of time from the second stimulus onward was relatively consistent in many cases. It is possible that the initial peak in flow was associated with a receptor-simulated event that was only slowly reversible, such as protein secretion and the fusion of secretory vesicles with the apical membrane. With the ability of our new method to collect pure samples lacrimal gland fluid, it should be possible to elucidate the temporal pattern of lacrimal protein secretion to determine whether it shows a similar early peak.

Our analysis of the ionic composition of lacrimal fluid is in good agreement with the earlier measurements published by Alexander et al. (1) in the rat and by Botelho and Martinez (2) in the rabbit. In the rat, Alexander et al. found values for $K^+$, $Na^+$, and $Cl^-$ of $46 \pm 3$, $135 \pm 5$, $123 \pm 1$ mM, while in the rabbit, Botelho and Martinez found that $K^+$, $Na^+$, and $Cl^-$ values were $42 \pm 4$, $107 \pm 4$, $126 \pm 5$ mM. The high $K^+$ values suggest substantial secretion of $K^+$ by the acinar cells into the lumen of the acini. This suggests that there are apically located $K^+$ channels, and immunocytochemistry using a rabbit antibody to the $\alpha$-subunit of the maxi-$K^+$ channels supports that conclusion (48). The high $Cl^-$ concentration also suggests apical $Cl^-$ channels. There are a number of patch-clamp studies showing that acinar cells have many $Cl^-$ channels (8, 39, 44), but there is little direct evidence for them to be located predominantly on the apical membrane (41). In the salivary gland, a similar location of $Cl^-$ channels was suggested (29). The concentrations of both $K^+$ and $Cl^-$ ions in the fluid in the lacrimal ducts suggest that there is movement of these ions across the apical membranes.

Furosemide reduced fluid secretion from stimulated intact lacrimal glands and blunted carbachol-induced cell shrinkage in isolated acinar cells. The inhibition was partial in both cases. The most plausible explanation for continuing secretion in the presence of furosemide is that acinar cells have other $Cl^-$ and $K^+$ transport systems that can move these ions into the cells. For example, $Cl^-$ can enter the cells via basolateral $Cl^--HCO_3^-$ exchangers (23). In many transporting epithelia, the $Cl^-\/HCO_3^-$ exchanger works in parallel with the $Na^+/H^+$ exchanger, which extrudes $H^+$ and prevents marked cellular acidification (32, 34, 36, 42). The net result is the uptake of $Cl^-$ and $Na^+$, and most of the transported $Na^+$ is exchanged for $K^+$, because $Na^+$ is extruded from the cytoplasm by the $Na^+$ pump. This process depends on cytosolic carbonic anhydrase, which has been identified in rabbit and rat (4) and rat and mouse lacrimal glands (18, 34).

In the isolated cells, exposure to furosemide alone during a 20-min period caused the cells to shrink slowly (Fig. 6C), an effect inhibited by a $Cl^-$ channel blocker (flufenamic acid).

This could be due either to furosemide affecting the uptake of $Cl^-$ ions by the cells or by furosemide blocking the exit of $Cl^-$, or both. Given that furosemide had little effect on the membrane currents induced by carbachol, it seems unlikely that the effect of furosemide is on the $Cl^-$ channels. This finding suggests that in these isolated cells, there is a leakage of $Cl^-$ from the cells that is replaced by an inward movement of $Cl^-$ due to a constitutively active NKCC1. If NKCC1 is blocked, the cytosolic pool of $Cl^-$ decreases and the cells shrink as a result of the decrease in total ion concentration within the cell. This would also suggest that the pool of $Cl^-$ ions available to move across the apical membrane is relatively small and is rapidly depleted on stimulation.

That furosemide produced significant inhibition, however, certainly supports the argument that NKCC1 is important for fluid production by the mouse lacrimal gland. It is an efficient transport system that moves one $K^+$ and two $Cl^-$ ions for each $Na^+$ ion that enters. In an in vivo perfusion system of rabbit lacrimal glands, it also has been shown that systemic furosemide significantly reduces carbachol-induced fluid delivery to the ocular surface (5), findings that suggest a similar role for the NKCC1 transporter in other species. In other cell types, stimulation results in activation of NKCC1 and/or upregulation of its expression (11, 13, 25). In some tissues, the decrease in intracellular $Cl^-$ ions that occurs upon cell activation stimulates NKCC1 (40). In the salivary gland, $\beta$-adrenergic activation of acinar cells results in upregulation of NKCC1 as a result of phosphorylation, although other agents that cause increased activity do not involve phosphorylation (45). In a human tracheal epithelial cell line, activation of NKCC1 is mediated via a protein kinase C (25, 26). Nitric oxide (NO) has been shown to inhibit NKCC1 activity in renal epithelial cells (17). Some nerves and acinar cells in mouse lacrimal glands have neural nitric oxide synthase (nNOS), which could produce NO and could result in NKCC1 inhibition and reduced fluid production (6). Increased nNOS activity or the failure of the normal control pathways to increase NKCC1 activity or expression would effectively reduce fluid production by the lacrimal gland and therefore could result in a dry-eye condition.

ACKNOWLEDGMENTS

We are grateful to Drs. R. J. Turner and E. Delpire for supplying the NKCC1 antibody. We are also grateful to Chris Picken for performing some of the cell volume measurements.

GRANTS

This work was supported by National Eye Institute Grant EY-014604.

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