Effects of Dexamethasone on Sodium–Potassium–Chloride Cotransport in Trabecular Meshwork Cells

Luanna K. Putney,* James D. Brandt,† and Martha E. O'Donnell*

Purpose. Previous studies in the authors’ laboratory have shown that bovine and human trabecular meshwork (TM) cells possess a robust sodium–potassium–chloride (Na–K–Cl) cotransport system that functions in regulating intracellular volume and may play a central role in modulating outflow facility across the TM. Dexamethasone, which can induce ocular hypertension, has been found to increase resistance to aqueous outflow across the TM. The current study was conducted to investigate the hypothesis that alteration of TM cell Na–K–Cl cotransport function, regulation, or both may be an underlying factor in steroid-induced glaucoma. To this end, the authors evaluated the effects of dexamethasone treatment of TM cells on Na–K–Cl cotransport activity and cotransporter protein expression.

Methods. Cultured bovine and human TM cell monolayers were exposed to dexamethasone (10⁻⁹ to 10⁻⁸ M) for varying times, then evaluated for Na–K–Cl cotransport activity or harvested for cellular membrane proteins. Cotransport activity was assessed as bumetanide-sensitive K influx. Cotransport protein expression was evaluated by Western blot analysis of cellular proteins using a monoclonal antibody to the human colonic T84 epithelial cell Na–K–Cl cotransporter.

Results. The authors found that 24- and 48-hour exposures of human and bovine TM cells to dexamethasone stimulates Na–K–Cl cotransport activity (10⁻⁸ to 10⁻⁶ M dexamethasone in human cells; 10⁻⁸ and 10⁻⁷ M in bovine cells). The authors also found that dexamethasone (10⁻⁸ M) stimulates Na–K–Cl cotransport activity of TM cells with exposure times as early as 12 hours and up to 5 days. In addition, the authors found that the level of Na–K–Cl cotransport protein expressed in TM cells is modulated by dexamethasone. When bovine or human TM cells are exposed to 10⁻⁸ or 10⁻⁶ M dexamethasone for 2 to 5 days, cotransporter protein expression is increased. With longer exposures, however, cotransporter protein levels decrease below control levels. Finally, the authors found that TM cells exposed to dexamethasone become unresponsive to regulation by hypertonicity and vasopressin.

Conclusions. The authors’ findings suggest that dexamethasone may be exerting its effect, at least in part, through altering Na–K–Cl cotransport function and regulation in TM cells. Invest Ophthalmol Vis Sci. 1997;38:1229-1240.
mediates Na, K, and Cl uptake into the cell in a 1:1:2 stoichiometry.\(^{19-21}\) The presence of the Na–K–Cl cotransporter has been documented in a variety of cell types. These studies have established the characteristic features of the cotransporter and also at least two of its functions. The Na–K–Cl cotransporter exhibits a high selectivity for Na, K, and Cl, has an absolute requirement for the presence of all three ion species, and is specifically inhibited by “loop” diuretics, such as furosemide, benzenzamide, and bumetanide. Activity of the Na–K–Cl cotransporter can be assessed as a bumetanide-sensitive (or bumetanide-inhibitable) K influx that is dependent on the presence of both Na and Cl. Our previous studies have shown that cultured bovine and human TM cells exhibit a robust K influx that is blocked by bumetanide and that this bumetanide-sensitive K influx is abolished when either Na or Cl (or both) is omitted from the assay medium. A characteristic of the cotransporter is that rubidium (Rb) can quantitatively substitute for K in this system (i.e., the cotransporter cannot distinguish between K and Rb). This is of experimental value because \(^{86}\)Rb has a half life of 18 days, whereas \(^{86}\)K has a half life of hours, making the latter impractical for use as a tracer. Thus, Na–K–Cl cotransport activity is assessed most often as a bumetanide-sensitive K influx, using \(^{86}\)Rb as a tracer for K (in assay medium containing Na, K, Cl, and trace amounts of \(^{86}\)Rb).

With respect to function, the cotransporter works in conjunction with the Na–K pump and Cl channels to perform vectorial transport of sodium chloride (NaCl) and water across both absorptive and secretory epithelia (i.e., kidney and intestine). The cotransporter also regulates intracellular volume of a variety of cell types, both epithelial and nonepithelial.\(^{19-21}\)

Work in our laboratory and elsewhere has provided evidence that Na–K–Cl cotransporter-mediated volume regulation of the endothelium is a major determinant of water flux across vessel walls.\(^{22-24}\) Our laboratory has shown recently the presence of high levels of Na–K–Cl cotransport activity and cotransporter protein expression in cultured bovine and human TM cells.\(^{25}\) We have shown that in bovine TM cells, the Na–K–Cl cotransporter plays a central role in regulation of intracellular volume and that its activity is modulated by extracellular toxicity and by a variety of hormones.\(^{25}\) Exposing TM cells to hypertonic medium immediately causes the cells to shrink as water is drawn from the intracellular space. Such treatment of the cells does not cause a loss of viability. Rather, the hypertonicity-induced cell shrinkage rapidly stimulates Na–K–Cl cotransport activity to mediate increased net uptake of Na, K, and Cl into the cells and, as water follows these ions, the cells reswell. TM cells are entirely dependent on the cotransporter for this cell reswelling, also called a regulatory volume increase, as inhibition of the cotransporter with bumetanide abolishes the restoration of cell volume.\(^{25}\) Exposing the cells to bumetanide itself also causes the cells to shrink but over a slower time course and in a manner that does not recover. Other means of blocking cotransporter activity, such as placing the cells in media lacking Na, K, or Cl, also cause a slow shrinkage of the cells. This suggests that the cotransporter functions to maintain TM cell volume even under isotonic conditions, most likely by offsetting Na, K, and Cl efflux pathways (such as the Na–K pump, K channels, and Cl channels). The TM Na–K–Cl cotransporter also appears to mediate hormone-driven changes in cell volume.\(^{25}\) Thus, in bovine TM cells, vasopressin, bradykinin, and other Ca-elevating and protein kinase C-activating hormones stimulate cotransport activity and swell the cells, whereas agents that elevate cyclic adenosine monophosphate or cyclic guanosine monophosphate inhibit the cotransporter and decrease TM cell volume. We also have found evidence that Na–K–Cl cotransport activity and intracellular volume are determinants of barrier function in cultured TM cell monolayers. That is, when bovine TM cells are cultured on permeable supports as a monolayer, the movement of \(^{14}\)C-sucrose across the monolayer and filter is influenced by Na–K–Cl activity and by intracellular volume.\(^{25}\) Exposures to hypertonic medium and bumetanide also increase permeability of the monolayer to \(^{14}\)C-sucrose; however, consistent with the effects on cell volume, hypertonicity causes an early increase in permeability that begins to decline after 10 minutes, whereas bumetanide causes a slower increase in permeability that continues to increase throughout the 15-minute assay. These findings support the hypothesis that intracellular volume of TM cells influences barrier function of the TM. A role for Na–K–Cl cotransport and TM cell volume in the regulation of aqueous outflow in the intact eye is further supported by the finding that exposing the TM of continuously perfused bovine and human anterior chambers to hypertonic medium results in an increased outflow facility, as does inhibition of the cotransporter by bumetanide (Al-Aswad et al, unpublished data, 1996) or Cl-free medium.\(^{26}\)

The glucocorticoid dexamethasone has been shown to alter the barrier function of the TM, decreasing outflow facility and inducing glaucoma. Given that the Na–K–Cl cotransporter plays such an important role in regulation of TM cell volume and given the evidence that TM cell volume may be a determinant of aqueous humor outflow facility, in the current study, we investigated the possibility that some of the outflow-reducing effects of dexamethasone may involve alteration of TM cell Na–K–Cl cotransport function or regulation or both. Specifically, we examined the effects of dexamethasone on Na–K–Cl cotrans-
port activity and cotransporter protein expression in both bovine and human TM cells. The results of the current study indicate that, indeed, the activity, expression, and regulation of the TM cell cotransporter are altered by dexamethasone.

METHODS

Cell Culture

Bovine TM cells were a gift of Dr. Beatrice Yue (Department of Ophthalmology, Lions of Illinois Eye Research Institute, Chicago, IL). Experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Human TM were isolated by methods based on those of Polansky et al. Tenets of the Declaration of Helsinki were followed, informed consent was obtained, and institutional human experimentation committee approval was granted for these studies. TM cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 5% calf serum, essential and nonessential amino acids, glutamine, and penicillin–streptomycin (TMC growth medium). Both types of TM cells were maintained in collagen-coated 75-cm² tissue culture flasks and were used between passages 3 and 7 (bovine) and 4 and 6 (human). Bovine aortic endothelial cells were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, glutamine, and penicillin–streptomycin. Bovine aortic endothelial cells were maintained in collagen-coated flasks and used between passages 3 and 12.

For experiments, cells were removed from flasks by brief trypsinization and were subcultured onto collagen-coated 24-well plates (transport assays) or onto collagen-coated 100-mm tissue culture dishes (Western blot analyses). TM cells were treated 5 to 7 days later as confluent monolayers with 10^-9 to 10^-6 M dexamethasone or carrier (0.1% ethanol in all cases) in TMC growth medium (containing 10% fetal bovine serum plus 5% calf serum). Serum was present during dexamethasone treatments to avoid loss of cell viability over longer treatment periods. Thus, both control and treated cells were exposed to growth medium containing serum for all treatment periods. Growth medium ± dexamethasone (or carrier) was replaced every day. TM cells treated with dexamethasone were found to proliferate more slowly than untreated cells; however, they still grew to confluence and there was no indication of reduced viability. For culturing in stock flasks, 24-well plates, and 100-mm dishes, all cells were maintained in a 95% air:5% carbon dioxide atmosphere.

Transport Measurements

Na–K–Cl cotransport was measured as bumetanide-sensitive K influx, using ^86Rb as a tracer for K. Details of this method have been published previously. Bovine or human TM cell monolayers on 24-well plates were equilibrated in an air atmosphere for 60 minutes at 37°C in an N2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes)-buffered minimal essential medium (MEM) containing (in millimolar): 144 Na, 147 Cl, 5.8 K, 1.2 Ca, 4.2 HCO3, 0.4 HPO4, 0.4 H2PO4, 0.4 Mg, 0.4 SO4, 5.6 glucose, and 20 Hepes. The cells were preincubated (5 or 7 minutes) with Hepes-buffered MEM containing 50 or 0 μM bumetanide. Cotransport then was assayed by replacing the preincubation medium with identical fresh medium containing 1 μCi/ml ^86Rb and incubating the cells again at 37°C for 5 or 7 minutes. In some cases, the preincubation and assay media contained vasopressin (100 nM) or were made hypertonic (400 mOsm) by the addition of NaCl. The assay was terminated by aspirating and rinsing the wells with ice-cold isotonic magnesium chloride to remove extracellular radioactivity. After a brief period of air drying, the contents of the wells were extracted with 1% sodium dodecyl sulfate and the amount of radioactivity present determined by use of a liquid scintillation counter (Tri-Carb Model 2500 TR; Packard Instruments, Downers Grove, IL). Osmolarities of all preincubation and assay media were verified by osmetry (model 3W2; Advanced Instruments, Norwood, MA). Samples of the sodium dodecyl sulfate extracts also were used to determine protein content by the bicinchoninic acid method. ^86Rb uptake was calculated as the slope of the linear phase of the uptake curve. ^86Rb uptake by way of Na–K–Cl cotransport was determined to remain linear for at least 10 minutes after addition of ^86Rb to the TM cells (data not shown). In the current study, ^86Rb uptake was determined over either a 5-minute or 7-minute assay period. Na–K–Cl cotransport was assessed as a bumetanide-sensitive K flux ([total K flux] – [K flux in the presence of bumetanide]). The K flux data are expressed as micromoles of K/gram of protein/minute.

Gel Electrophoresis and Western Blotting

Cell monolayers were washed with ice-cold phosphate-buffered saline (PBS)/2 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4) containing the following protease inhibitors: phenylmethylsulfonyl fluoride (20 μg/ml), N-tosyl-L-phenylalanine chloromethyl ketone (88 μg/ml), pepstatin A (1 μg/ml), chymostatin (2 μg/ml), leupeptin (1.25 μg/ml), aprotinin (6.3 U/ml), and 4-(2-aminoethyl)benzenesulfonyl fluoride (1.25 μg/ml). Cells then were scraped from dishes and centrifuged at 7500g for 10 minutes at 4°C. The cell pellet was resuspended in 0.2 to 1 ml protease inhibitor-containing PBS–EDTA, and the suspension passed through a 1-ml, 26-gauge 1/2 syringe several times. Cells then were sonicated for 30 seconds at 4°C.
(XL2020 Sonicator of Heat Systems, Farmingdale, NY) and cellular debris removed by brief centrifugation at 9500g and 4°C. The supernatant then was centrifuged at 150,000g for 40 minutes. The resulting crude membrane preparations were resuspended in the protease inhibitor-containing PBS-EDTA. For whole cell lysates, cells were scraped into protease inhibitor-containing PBS-EDTA and lysed by sonication, then subjected to electrophoresis, omitting the ultra-centrifugation step. Regardless of whether membrane preparations or cell lysates were used, Western blot analyses gave similar results (see Results). For both whole cell lysates and crude membrane preparations, protein contents were determined before electrophoresis by the bicinchoninic acid method.\textsuperscript{20}

Cellular lysates, membrane protein samples, and pre-stained molecular mass markers (Bio-Rad, Hercules, CA) were denatured in sodium dodecyl sulfate reducing buffer [9.2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 50 mM tris(hydroxymethyl)aminomethane (Tris)/HCl, pH 7.4, 35% sucrose, and 0.012% bromophenol blue] and were heated at 100°C for 3 minutes. The samples then were separated electrophoretically by polyacrylamide gel electrophoresis using gels containing 6% or 8% polyacrylamide (Mini-PROTEAN II; Bio-Rad). Equivalent amounts of total cell lysate protein or total crude membrane protein were added to each lane of the gel (either 20, 25, or 30 μg total protein). Samples then were electrophoresed at 150 V for 1 hour and the resolved proteins of other mammalian cells.\textsuperscript{31} Details of methods using these antibodies for Western blot analysis have been published by O’Donnell et al\textsuperscript{32} and Sun et al.\textsuperscript{33}

Materials

Bumetanide was purchased from ICN Biomedicals (Costa Mesa, CA).\textsuperscript{88}Rb\textsuperscript{+} was obtained from Du Pont–New England Nuclear (Boston, MA), vasopressin was purchased from Peninsula Laboratories (Belmont, CA), and dexamethasone was obtained from Sigma Chemical (St. Louis, MO). Eagle’s minimal essential medium was purchased from JRH Biosciences (Lenexa, KS), and fetal bovine serum and calf serum were from HyClone Laboratories (Logan, UT). Collagen (type 1) was from Collaborative Research (Bedford, MA).

Statistical Analysis

Statistical analyses were performed using Student’s t-test for unpaired data.

RESULTS

Effects of Dexamethasone on Na–K–Cl Cotransport Activity and Cotransporter Protein Expression in Bovine Trabecular Meshwork Cells

To test whether dexamethasone influences Na–K–Cl cotransport activity in bovine TM cells, we first examined Na–K–Cl cotransport activity after exposure of bovine TM cells to varying concentrations of dexamethasone, as shown in Figure 1. Dexamethasone treatment of the cells caused significant elevation of cotransport activity in cultured bovine TM cells at concentrations of $10^{-8}$ and $10^{-7}$ M but not at $10^{-6}$ M. Specifically, we found that Na–K–Cl cotransport activity, assessed as bumetanide-sensitive K influx, increased after 24-hour treatment with dexamethasone from the control level of $13.93 \pm 0.62$ to $20.95 \pm 0.59$ and $16.42 \pm 1.06$ μmol K/g protein/min ($10^{-8}$ and $10^{-7}$ M dexamethasone, respectively).

In these studies, we also evaluated bumetanide-sensitive K influx after exposing bovine TM cells to $10^{-8}$ M dexamethasone (or control medium lacking dexamethasone) for varying times. The results shown in Figure 2 indicate that $10^{-8}$ M dexamethasone caused a 17% increase in Na–K–Cl cotransport activity after a 1-day exposure and increases of 26%, 56%, and 69% after exposures of 2 days, 3 days, and 5 days, respectively. The Na–K–Cl cotransport was stimulated significantly by dexamethasone with an exposure time as early as 12 hours (data not shown). With an exposure of 7 days, cotransport activity was elevated only 20%, and at longer exposure times up to 14 days, no
significant elevation of cotransport activity above the control level was seen.

We have shown previously by Western blot analysis that a protein of approximately 170 kDa is recognized in several types of endothelial cells by a monoclonal antibody (T4) to the human colonic T84 epithelial cell Na–K–Cl cotransporter.\textsuperscript{12,33} Figure 3 shows that in addition to recognizing the bovine aortic endothelial cell cotransporter, the T4 monoclonal antibody also recognizes the Na–K–Cl cotransporter protein in both bovine and human TM cells (BTMC and HTMC, respectively) and that this protein also is approximately 170 kDa in size.

To examine whether the dexamethasone-induced stimulation of Na–K–Cl cotransport activity is mediated by upregulation of cotransporter protein expression, we evaluated the amount of Na–K–Cl cotransporter protein in TM cells after dexamethasone exposure over a course of 12 days. Figure 4 shows that dexamethasone (10^{-8} M) induced a transient increase followed by a marked decrease in expression of the 170-kDa cotransporter protein in bovine TM cells over time. The relative amount of cotransporter protein expression of several experiments was quantitated by densitometric analysis for comparison to dexamethasone-induced temporal changes in cotransporter activity. The results of these analyses are shown in Figure 5 as filled symbols. In these experiments, cotransporter protein expression (plotted as relative band density) increased by 41\% after 3 days' exposure to 10^{-8} M dexamethasone, but then decreased by 17\% after 5 days' exposure and further decreased by 63\% after 12 days' exposure. Figure 5 also summarizes the relative Na–K–Cl cotransport activity in bovine TM cells after varying exposure times to 10^{-8} M dexamethasone. These data indicate that the dexamethasone-induced stimulation of Na–K–Cl cotransport activity, which peaks at 5 days' exposure, is accompanied by a commensurate increase in cotransporter protein expression. However, with longer dexamethasone exposure times, Na–K–Cl cotransport returns to control levels, whereas cotransporter protein expression is reduced dramatically below that of control levels. The signifi-
Effects of Dexamethasone on Na-K-Cl Cotransport Activity and Cotransporter Protein Expression in Human Trabecular Meshwork Cells

To investigate whether dexamethasone also influences Na-K-Cl cotransport activity in human TM cells, we examined bumetanide-sensitive K influx after exposure of these cells to varying concentrations of dexamethasone. As shown in Figure 6, we found that cotransport activity of cultured human TM cells was elevated significantly after 48-hour exposure to $10^{-8}$ to $10^{-6}$ M dexamethasone. Human TM cells exhibited a bumetanide-sensitive K influx of $11.27 \pm 0.48 \mu$mol K/g protein/min, which was increased after exposure to $10^{-8}$, $10^{-7}$, and $10^{-6}$ M dexamethasone to $14.31 \pm 0.54$, $14.47 \pm 0.68$, and $13.04 \pm 0.25 \mu$mol K/g protein/min, respectively. No significant elevation of cotransport activity was observed with $10^{-9}$ M dexamethasone. In addition, we found that cotransport activity in these cells was elevated significantly after 24-hour exposure to $10^{-7}$ and $10^{-6}$ M dexamethasone, but not after exposure to $10^{-9}$ or $10^{-8}$ M (data not shown).

To determine whether dexamethasone influences Na-K-Cl cotransporter protein expression in human TM cells, we examined the effect of 2-day dexamethasone exposure on the level of cotransporter protein in these cells compared with that of untreated cells. The results shown in Figure 7 indicate that $10^{-6}$ M dexamethasone caused a marked increase in cotransporter protein expression after 2 days’ exposure. At longer dexamethasone exposure times, we found that cotransporter protein expression was reduced greatly in the human TM cells from that of control level (by 49% after 12-day exposure; data not shown). This effect was similar to that seen in bovine TM cells.

Effects of Dexamethasone on Regulation of Na-K-Cl Cotransport Activity in Trabecular Meshwork Cells

As an initial approach to determine whether dexamethasone alters the regulation of the Na-K-Cl cotransporter system in TM cells, we evaluated the ability of the cotransporter to respond to agents that we have shown previously to be regulators of the TM cotransporter. Specifically, in these experiments, we investigated the response of bovine TM cell bumetanide-resistant Na-K-Cl cotransport system to dexamethasone.
Modulation of TM Na–K–Cl Cotransport by Dexamethasone

FIGURE 5. Relative dexamethasone effects on sodium–potassium–chloride (Na–K–Cl) cotransport activity and cotransporter protein expression in bovine trabecular meshwork cells. Cotransport activity values shown are data of Figure 2 replotted to illustrate relative changes in Na–K–Cl cotransport activity (bumetanide-sensitive K influx; open circles) after exposure of bovine trabecular meshwork cells to 10⁻⁸ M dexamethasone. Cotransporter protein expression is shown as relative band density values and includes data of Figure 4 (filled squares) plus one additional experiment (filled circles). The dexamethasone-induced stimulation of Na–K–Cl cotransport activity, which peaks at 5-day exposure, was accompanied by an increase in cotransporter protein expression. However, with longer dexamethasone exposure times, Na–K–Cl cotransport returned to control levels, whereas cotransporter protein expression was reduced well below control levels.

In a separate set of experiments, we examined the effect of dexamethasone on the ability of the cotransporter to be stimulated by vasopressin. Thus, cells were exposed to 0 or 10⁻⁸ M dexamethasone for 1 to 12 days, then assayed for bumetanide-sensitive K influx in the presence or absence of vasopressin. As shown in Figure 9, in cells not pretreated with dexamethasone, exposure to vasopressin (100 nM) for 10 minutes (5 minutes preincubation and 5 minutes assay) increased cotransport activity significantly from a control level of 14.03 ± 1.01 to 23.24 ± 1.53 μmol K/g protein/min. However, when TM cells were exposed to 10⁻⁸ M dexamethasone for 1 to 12 days, the vasopressin stimulation of cotransport activity was abolished. At exposure times of 1, 3, and 5 days, exposure to vasopressin reduced activity significantly to even below dexamethasone-treated control values, consistent with the observed effects of hypertonic media in cells treated with dexamethasone for 5 days (Fig. 8). These data suggest that dexamethasone alters the ability of the Na–K–Cl cotransporter to respond appropriately to regulatory factors such as hypertonicity and vasopressin.

DISCUSSION

Our findings indicate that dexamethasone alters activity of the TM cell Na–K–Cl cotransporter as well as sensitive K influx to two potent stimulators of cotransport activity: extracellular hypertonicity and vasopressin. Cultured bovine TM cells were pretreated with 0 or 10⁻⁸ M dexamethasone for 1 to 12 days, then assayed for bumetanide-sensitive K influx in either isotonic (290 mOsm) or hypertonic (390 mOsm) media. As shown in Figure 8, in cells not pretreated with dexamethasone (0 days' exposure), hypertonic medium significantly increased Na–K–Cl cotransport activity from the control level of 17.77 ± 0.58 to 23.35 ± 0.76 μmol K/g protein/min (31% increase). However, when TM cells were exposed to 10⁻⁸ M dexamethasone for varying times, the hypertonicity-induced stimulation of the Na–K–Cl cotransporter was either diminished (1 or 3 days' exposures) or abolished (5 or 12 days' exposures). These data suggest that dexamethasone alters the ability of the Na–K–Cl cotransporter to respond appropriately to elevated extracellular tonicity. Moreover, in cells exposed to dexamethasone for 5 days, cotransport activity actually was significantly lower in hypertonic than in isotonic medium. This will be considered further in the Discussion section.

In a separate set of experiments, we examined the effect of dexamethasone on the ability of the cotransporter to be stimulated by vasopressin. Thus, cells were exposed to 0 or 10⁻⁸ M dexamethasone for varying times, then assayed for bumetanide-sensitive K influx in the presence or absence of vasopressin. As shown in Figure 9, in cells not pretreated with dexamethasone, exposure to vasopressin (100 nM) for 10 minutes (5 minutes preincubation and 5 minutes assay) increased cotransport activity significantly from a control level of 14.03 ± 1.01 to 23.24 ± 1.53 μmol K/g protein/min. However, when TM cells were exposed to 10⁻⁸ M dexamethasone for 1 to 12 days, the vasopressin stimulation of cotransport activity was abolished. At exposure times of 1, 3, and 5 days, exposure to vasopressin reduced activity significantly to even below dexamethasone-treated control values, consistent with the observed effects of hypertonic media in cells treated with dexamethasone for 5 days (Fig. 8). These data suggest that dexamethasone alters the ability of the Na–K–Cl cotransporter to respond appropriately to regulatory factors such as hypertonicity and vasopressin.
FIGURE 7. Effect of dexamethasone on sodium-potassium-chloride (Na-K-Cl) cotransport protein expression in human trabecular meshwork cells (HTMCs). HTMCs were exposed to 10^{-6} M dexamethasone for 48 hours, then subjected to Western blot analysis of Na-K-Cl cotransport protein using T4 monoclonal antibody. Proteins from membrane preparations were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (either 20 or 30 μg loaded in each lane), transferred to nitrocellulose, and probed with T4 monoclonal antibody as described in the Methods section. The blot was visualized by enhanced chemiluminescence assay. Dexamethasone induced a marked increase in cotransporter protein expression after 2-day exposure in HTMCs. Data are from a representative experiment. One other experiment produced similar results.

the level of cotransporter protein expression, both in bovine and human cultured TM cells. Exposure of TM cells to 10^{-8} M dexamethasone causes a transient increase in cotransporter activity that peaks at 5 days, then returns to control levels by 8 days of exposure. Cotransporter protein expression also is increased by dexamethasone treatment of the cells, with the peak effect observed around 3 days of exposure and with protein expression falling well below control levels thereafter. Thus, it appears that although longer exposures to dexamethasone cause a reduction in the amount of cotransporter protein present in the cells, bumetanide-sensitive K influx relative to the amount of cotransporter protein is increased. This indicates that the fewer cotransporters present are operating at a higher rate (i.e., faster K uptake per cotransporter protein), resulting in maintenance of cotransport activity at or near control levels. One explanation for this finding is that regulation of cotransport activity may be altered by dexamethasone treatment. That is, the cotransporters are more activated under basal conditions in the absence of any stimulatory agent such as hypertonicity or vasopressin. This may reflect an uncoupling of the cotransporter from normal regulatory influences that determine activity of the cotransporter (i.e., dexamethasone may cause the cotransporter to be in an activated state that is uncoupled from normal regulation). An alternate explanation is that dexamethasone induces expression of an isoform of the Na-K-Cl cotransporter that is not recognized by the monoclonal antibody, such that the drop in cotransporter protein after prolonged dexamethasone exposure is only an apparent decrease. To date, only two forms of the cotransporter have been identified. These are NKCC1, a widespread form found in secretory epithelial cells and also vascular endothelial cells, and NKCC2, the form found in absorptive renal epithelial cells.31,34 The monoclonal antibody used in our studies is known to recognize both NKCC1 and

FIGURE 8. Effect of dexamethasone on hypertonicity-induced stimulation of the sodium-potassium-chloride (Na-K-Cl) cotransporter in bovine trabecular meshwork cells. Cells were pretreated for 0 to 12 days with 10^{-8} M dexamethasone in growth medium, as described in the Methods section. Bumetanide-sensitive K influx of the cells then was assessed in either isotonic (control level) or hypertonic assay media. To do this, cells were preincubated for 5 minutes in a Hepes-buffered medium that was either isotonic (290 mOsm) or hypertonic (390 mOsm, by addition of NaCl) and also contained either 0 or 50 μM bumetanide. For assay of cotransport activity, cells then were incubated for 5 minutes in media identical to the preincubation media with the exception that 86Rb (1 μCi/ml) also was present. Cells not pretreated with dexamethasone, hypertonic medium significantly increased Na-K-Cl cotransport activity over the control level. However, when TM cells were exposed to dexamethasone for varying times, the hypertonicity-induced stimulation of the cotransporter was either diminished (1- or 3-day exposure) or abolished (5- or 12-day exposure). Data are mean values ± standard error of the mean, n = 8. Asterisks indicate significant differences at P < 0.05 (*) and at P < 0.001 (**) by Student's t test.
may no longer be able to reswell the cells after they have been shrunken by hypertonicity. In addition, the finding that dexamethasone abolishes vasopressin-induced stimulation of cotransport activity suggests that the steroid blocks cotransporter-mediated, hormone-driven changes in TM cell volume. Although hypertonicity and vasopressin are only two regulators of TM cell cotransport activity, the response of the TM cotransporter to these different stimulators serves as an index of the cotransporter’s ability to be modulated by environmental signals. The finding that longer dexamethasone exposure times increase the activity of the cotransporter relative to the amount of cotransporter protein present in the cells also is consistent with an uncoupling of the cotransporter from its normal regulatory influences. That is, dexamethasone appears to stimulate the cotransporter and render it insensitive to modulation by hypertonicity and vasopressin. Because regulation of TM cell volume is critically dependent on the ability of the cotransporter to respond to these environmental signals, our findings suggest that dexamethasone impairs the TM cell’s ability to volume regulate appropriately.

In TM cells exposed to dexamethasone for shorter periods, we observed that hypertonicity (after 5 days of dexamethasone exposure) and vasopressin (after 1 to 5 days of dexamethasone exposure) actually reduced cotransport activity to below the dexamethasone-treated control levels. We currently do not know the cellular mechanisms that underlie this finding. However, we do know that the signal transduction pathways that influence activity of the cotransporter are complex, that they include activities of kinases and phosphatases, and that phosphorylation of the cotransporter protein appears to play a role in regulating its activity. It is possible that dexamethasone induces changes in the relative expression and activity of the cells’ signal transduction components, such as kinases or phosphatases or both, which, in turn, influence the cotransporter’s response to regulators, such as vasopressin and hypertonicity.

It is well known that glucocorticoids can elevate IOP and induce glaucoma in susceptible persons. It also has been documented that cultured human TM cells do not respond equally to glucocorticoid treatment. In the current studies, we have found some variation in the magnitude of the cotransporter activity and protein expression responses to dexamethasone in human TM cells; however, our studies have not used a sufficiently large population to address this issue adequately.

It has been shown that in a subset of the population, glucocorticoid treatment can elevate IOP after both long-term exposure and acute exposures as brief as 4 to 8 hours. Elevation of IOP with dexamethasone also has been observed in a human organ-cul-
ture system after only 10 days of exposure. Dexamethasone further has been shown to induce a variety of cellular changes in cultured human TM cells within 1 to 14 days of exposure, including changes in extracellular matrix production, cell proliferation, and cell size; alteration of the cytoskeleton; and decreased hydraulic conductivity. In addition, dexamethasone has been shown to alter ion transport through Cl channels, K channels, and the Na/H antiporter in a variety of nonocular cell types within hours of exposure. Our findings indicate that dexamethasone induces acute increases in both Na–K–Cl cotransporter activity and protein expression (peaking at 2 to 5 days' exposure) but, with longer exposures, causes a return of cotransport activity to basal levels concomitant with a notable fall in the amount of cotransporter protein present in the cells and a loss of cotransporter responsiveness to its regulators.

There is much evidence to suggest that dexamethasone affects the morphology of cultured TM cells, including changes in cytoskeletal organization and cell size. Clark et al have described the generation of glucocorticoid-induced cross-linked actin networks, which begin to form after 3 to 4 days of dexamethasone exposure and continue to accumulate over several weeks of exposure. In this regard, there is increasing evidence to suggest a role for the cytoskeleton in regulation of ion transport processes. For example, rearrangement of microfilaments appears to be involved in the activation of the Na–K–Cl cotransporter in human colonic T84 epithelial cells. Thus, it is possible that the initial reorganization of TM cell microfilaments induced by dexamethasone contributes to the transient increase in Na–K–Cl cotransport activity that we observe. Similarly, it is possible that dexamethasone-induced alteration of the TM cell cytoskeleton contributes to the apparent uncoupling of the Na–K–Cl cotransporter from its regulation by vasoressin and hypertonicity. An increase in TM cell size, measured by morphometric analysis of cell area, also has been reported to occur with dexamethasone exposure. Consistent with this, in the current study, we found that bovine TM cells treated with dexamethasone had a more spread morphology by 14 days' exposure. However, in preliminary studies conducted to evaluate the effects of dexamethasone on TM cell volume using 14C urea and 14C sucrose as markers for total and extracellular water space, respectively, we did not find a significant change in TM cell volume after dexamethasone exposure (data not shown). It is possible that dexamethasone causes a flattening of TM cells, giving the appearance of increased intracellular volume, without an actual increase in volume occurring. Whether dexamethasone alters TM cell volume responses to hypertonicity and hormones is the subject of ongoing investigations in our laboratory.

We do not yet know the molecular identity of the TM cell Na–K–Cl cotransporter. The two known forms of the cotransporter, NKCC1 and NKCC2, both appear to function in vectorial transport of NaCl across epithelia. NKCC1, however, also has been shown to function in cell volume regulation. Northern blot analyses have provided evidence that the NKCC1 form has a widespread tissue distribution. It has been hypothesized that NKCC1 may serve as the housekeeping form of the cotransporter, functioning to regulate intracellular volume and perhaps also intracellular Cl concentration in addition to vectorial ion transport in epithelium. We have found recently that the bovine aortic endothelial cell Na–K–Cl cotransporter, which is functionally similar to the TM cell cotransporter, is of the NKCC1 type. Current studies are underway to identify the molecular form and tissue distribution of the TM cell Na–K–Cl cotransporter.

It is well established that the TM provides a dynamic barrier to aqueous humor outflow and that the resistance of the barrier is modulated precisely. There is increasing evidence that exposure of the TM to dexamethasone impairs the ability of the barrier to be modulated appropriately. Our previous studies have shown that the Na–K–Cl cotransporter plays an important role in regulating intracellular volume of cultured TM cells and that permeability of cultured TM cell monolayers is increased by hypertonicity and by bumetanide, both of which cause a decrease in TM intracellular volume. We hypothesize that changes in TM cell volume, mediated by changes in Na–K–Cl cotransport activity, play a role in modulating facility of aqueous humor outflow in intact TM. Consistent with this hypothesis are the findings by Al–Aswad et al that hypertonic solutions, Cl-free solutions, and bumetanide (Al–Aswad et al, unpublished data, 1996), all of which reduce TM cell volume, cause an increase in outflow facility of perfused anterior chamber preparations. The results of the current study, together with these previous observations, suggest that TM cell volume is a determinant of TM barrier resistance and that the increased resistance observed with dexamethasone treatment may involve changes in the function and expression of the Na–K–Cl cotransporter.

Key Words
anterior segment, cultured cells, glaucoma, glucocorticoids, membrane transport, trabecular meshwork

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Modulation of TM Na–K–Cl Cotransport by Dexamethasone

References


