Education

ANSWERS

1. What is the pathophysiology of SLK?

Since Theodore published the first clinical description of SLK in 1963,[1] numerous aetiologies have been proposed for this specific keratoconjunctivitis.[2] Although the aetiology of SLK has not been definitively established, the authors hold the mechanical friction theory to be most plausible.

The mechanical friction theory originally advocated by Wright[3] is based on a characteristic feature of SLK, redundancy and loosening of the superior bulbar conjunctiva. The mechanical theory suggests that the superior bulbar conjunctiva is continually rubbed by the upper tarsus during blinking, resulting in chronic inflammation. This theory is supported by the clinical observations that SLK tends to be associated with thyroid dysfunction and with the use of hydrophilic contact lenses.[4 5] SLK may also be present in eyes with essential blepharospasm and eyes with previous upper lid blepharoplasty.[6] Thus, the pathophysiology of SLK appears to involve abnormal dynamics or an abnormal interface load between the eyelid and the globe, resulting in friction on blinking that leads to chronic irritation and the development of keratoconjunctivitis.[7]

2. What medical treatment options exist for SLK?

A number of medical treatment modalities have been proposed for SLK.[2] The local application of silver nitrate, originally recommended by Theodore,[1] appears to facilitate scarring and remodelling of the subconjunctival tissue, which in turn is proposed to decrease friction between the bulbar and palpebral conjunctiva. Bandage contact lenses, which are particularly effective in treating eyes with filamentary keratitis, are thought to exert a therapeutic effect by isolating the globe mechanically from the motion of the tarsus.[8]

Pre-existing dry eye is linked to SLK,[1 4] indicating that the loss of lubricity may contribute to the development of SLK. Lubricating eye-drops including artificial tears, sodium hyaluronate, and autologous serum, therefore, may be of value to some degree.[9] Occlusion of the upper and/or lower puncta may be effective by increasing the amounts of tears that facilitate lubrication of the ocular surface.[10] Pharmacological therapies intended to lesson ocular surface inflammation may also be considered. Corticosteroid, ciclosporin A and cromolyn sodium have been used for this purpose.[5 11] In general, however, the effects of medical treatments are limited, and when they fail, surgical treatments become necessary.

3. What surgical options exist for SLK?

Thermocautery,[10] simple resection[2 12] and recession of the abnormal conjunctiva[13] have been reported as effective surgical treatments for SLK. New surgical methods such as a crescent resection of the superior unaffected bulbar conjunctiva[14] and amniotic membrane transplantation[6] have recently been proposed. Additionally, in this case report, we describe the use of conjunctival fixation sutures, which, to our knowledge, is a novel approach for the treatment of SLK.

Surgical options for the treatment of SLK can be divided into two categories based on the mechanism of action, procedures that seek to reinforce the adhesion of the conjunctiva to the sclera and those that seek to correct the redundancy of the superior bulbar conjunctiva. Procedures in the first category include thermocautery, recession of the abnormal conjunctiva and amniotic membrane transplantation. Procedures in the latter category include resection of the abnormal conjunctiva, or resection of the unaffected superior bulbar conjunctiva, as well as the conjunctival fixation suture method described in this case report.

DISCUSSION

SLK, an inflammatory disease involving the region of the limbus and the superior bulbar conjunctiva, may result in conditions such as filamentary keratitis, superficial punctate keratopathy and hyperaemia of the limbus and superior bulbar conjunctiva.[2 4] There is prominent laxness of the superior bulbar conjunctiva when the upper lid was squeezed in patients with SLK. Recently, Yokoi *et al*[14] and Kheirkhah *et al*[6] independently reported that a redundant, loosened superior bulbar conjunctiva plays a significant role in the pathogenesis of SLK. These authors advocated categorising SLK as a type of conjunctivochalasis, or recognising SLK as superior conjunctivochalasis.[6 14]

Based in part on our successful experience using fixation sutures for the treatment of conjunctivochalasis, a method which was originally reported by Otaka and Kyu,[15] we accordingly chose a similar technique for the treatment of refractory SLK. Though many surgical procedures to treat SLK have been investigated, fixation sutures offer the unique benefit of simple, quick, and minimally invasive application. Additionally, a large area of the superior bulbar conjunctiva remains unaffected by this procedure, which may be beneficial in the event of future ocular surgery, such as cataract or glaucoma surgery. We have now successfully treated a total of three patients with refractory SLK with the procedure described in this case report.

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The Effects of Dexamethasone on the Na,K-ATPase Activity and Pump Function of Corneal Endothelial Cells

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ABSTRACT

Purpose: The Na^+- and K^+-dependent ATPase (Na,K-ATPase) expressed in the basolateral membrane of corneal endothelial cells plays an important role in the pump function of the corneal endothelium. We investigated the possible role of dexamethasone in the regulation of Na,K-ATPase activity and pump function in corneal endothelial cells. *Methods:* Confluent monolayers of mouse corneal endothelial cells were exposed to dexamethasone. ATPase activity of the cells was evaluated by spectrophotometric measurement of phosphate released from ATP with the use of ammonium molybdate, with Na,K-ATPase activity being defined as the portion of total ATPase activity sensitive to ouabain. Pump function of the cells was measured with the use of an Ussing chamber, with the pump function attributable to Na,K-ATPase activity being defined as the portion of the total short-circuit current sensitive to ouabain. Western blot analysis was examined to measure the expression of the Na,K-ATPase α_1-subunit. *Results:* Dexamethasone (1 or 10 μM) increased the Na,K-ATPase activity and pump function of the cultured cells. These effects of dexamethasone were blocked by cycloheximide, a protein synthesis inhibitor. Western blot analysis also indicated that dexamethasone increased the expression of the Na,K-ATPase α_1-subunit, whereas it decreased the expression of the phospho-Na,K-ATPase α_1-subunit. *Conclusions:* Our results suggest that dexamethasone stimulates Na,K-ATPase activity in mouse corneal endothelial cells. The effect of dexamethasone activation in these cells is mediated by Na,K-ATPase synthesis and increase in an enzymatic activity by dephosphorylation of Na,K-ATPase α_1-subunits.

Keywords: Corneal endothelial cells; dexamethasone; Na,K-ATPase; protein synthesis; short-circuit current

A single layer of endothelial cells covers the posterior surface of Descemet's membrane of the cornea in a well-arranged mosaic pattern.[1] Corneal hydration is determined primarily by the balance between the penetration of aqueous humor across the corneal endothelium into the stroma and the subsequent pumping of the fluid out from the stroma. The accumulation of fluid in the stroma resulting from disturbance of this balance may lead to bullous keratopathy, which is characterized by an edematous cornea with a reduced transparency.[2]

Total pumping activity for the removal of fluid from the cornea is determined by the number of endothelial cells and the pump function of each cell. Given that human corneal endothelial cells have a limited proliferative capacity, endothelial dystrophies, ocular trauma, corneal graft rejection, and insults associated

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with intraocular surgeries may result in corneal endothelial cell loss and permanent damage. Replacement of the corneal endothelium by keratoplasty is currently the only established therapeutic approach to recovery of endothelial cell number. Pseudophakic or aphakic bullous keratopathy, Fuchs' endothelial dystrophy and failed corneal grafts remain common indications for keratoplasty, accounting for ~60% of the total number of such procedures.[3–5]

Activation of the pump function of remaining endothelial cells is a potential alternative approach to recovery of the total pumping activity of the cornea, so long as the total number of such cells is within an acceptable range. However, therapeutic approaches to the activation of corneal endothelial cells remain to be established.

The Na^+- and K^+-dependent ATPase (Na,K-ATPase) expressed in the basolateral membrane of corneal endothelial cells is primarily responsible for the pump function of the corneal endothelium.[1] The Na,K-ATPase pump site density in the corneal endothelium was found to be increased in eyes affected by moderate guttata,[6] whereas it showed an initial increase, a sudden marked decrease, and a subsequent gradual decline associated with the end stage of disease in patients with Fuchs' endothelial dystrophy.[7] These observations indicate that certain conditions can induce a compensatory increase in Na,K-ATPase pump site density and, presumably, in endothelial pump function. Thus, they also suggest the existence of a regulatory mechanism (or mechanisms) for control of total Na,K-ATPase activity in the corneal endothelium.

Several studies have shown that glucocorticoids stimulate Na,K-ATPase activity through multiple complex mechanisms, including gene expression, transcription, translocation, and enzymatic activity in a variety of tissues.[8] Although the results of experimental studies concerning the effects of glucocorticoids on corneal endothelial damage do not show any close correlations,[9–11] topical glucocorticoids have been clinically used for the treatment of corneal endothelial disorders. Indeed, we previously showed that steroid administration appeared to increase endothelial pump function and ameliorate stromal edema in a patient with bullous keratopathy secondary to Sato's refractive surgery.[12] These observations led us to the idea that the Na,K-ATPase activity and pump function of the corneal endothelium may be upregulated by glucocorticoids.

We have now investigated the role of dexamethasone in controlling Na,K-ATPase in corneal endothelial cells. We thus examined the effects of dexamethasone on the enzymatic activity and pump function of the Na,K-ATPase in cultured mouse corneal endothelial cells. In addition, we also examined the mechanisms by which dexamethasone might affect Na,K-ATPase activity with the use of cycloheximide, a protein synthesis inhibitor, and with Western blot analysis of the Na,K-ATPase α_1-subunit.

MATERIALS AND METHODS

Chemicals

Ammonium molybdate reagent (Biomol Green) and phosphate standards were obtained from Biomol Research Laboratories (Plymouth, PA, USA). M-PER Mammalian Protein Extraction Reagent and Pierce BCA Protein Assay Kit were obtained from Pierce Biotechnology (Rockford, IL, USA). Anti-Na/K ATPase α_1 antibody was obtained from Cosmo Bio (Tokyo, Japan). Phospho-Na,K-ATPase α_1-subunit is considered to be the inactive state of the Na,K-ATPase α_1-subunit, and anti-phospho-Na/K-ATPase α_1 (Ser-18) antibody was obtained from Cell Signaling (Danvers, MA, USA). Anti-β-actin antibody (AC-15) was obtained from Abcam (Cambridge, MA, USA). Vectastain Elit ABC Rabbit IgG Kit was obtained from Funakoshi (Tokyo, Japan). ECL Plus Western Blotting Detection Reagent was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Dexamethasone, cycloheximide, ouabain, and other chemicals were obtained from Sigma (St. Louis, MO, USA). Water insoluble compounds were dissolved in a minimal volume of methanol or dimethyl sulfoxide, with equal amounts of these solvents being added to control solutions; the final concentration of methanol or dimethyl sulfoxide in incubations was <0.3% and was found to have no effect on ATP hydrolysis or short-circuit current (data not shown).

Cell Culture

A simian virus 40 (SV40)-transformed mouse corneal endothelial cell line (C3H) was kindly provided by J. W. Streilein (Harvard Medical School). The cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were passaged at a split ratio of 1:2 to 1:4, at which they become confluent in 4 to 7 days. Experiments examining PKC activity or Na,K-ATPase activity were performed with cells cultured in 24-well culture plates (Nunc, Roskilde, Denmark). Ussing chamber experiments were performed with confluent monolayers of cells cultured on 6-well polycarbonate Snapwell inserts

(Corning, Acton, MA, USA) with a membrane pore size of 0.4 μm. Snapwell inserts membrane growth area was 4.67 cm^2. All experiments were performed with cell monolayers within one day of the cells reaching confluence and with the cells maintained in the culture incubator at 37°C.

Measurement of Na,K-ATPase Activity

The culture medium was removed from cell monolayers, ultrapure distilled water (150 μl) was added to each well, and the culture plate was then placed in liquid nitrogen for 10 sec before the addition to each well, at room temperature, of 150 μl of a solution containing 80 mM histidine, 20 mM KCl, 6 mM $MgCl_2$, 2 mM EGTA, alamethicin (2 μg/ml), 30 μM digitonin, and 200 mM NaCl at pH 7.4.[13] To duplicate wells, 10 μl of 30 mM ouabain (final concentration, 1 mM) or vehicle was added, and the plate was incubated for 30 min at 37°C. After the further addition of 10 μl of 300 mM ATP (final concentration, 10 mM), the reaction mixtures were incubated for an additional 30 min at 37°C. The ATP hydrolysis reaction was terminated by the addition of 75 μl of 50% trichloroacetic acid to each well. The contents of each well were then centrifuged at 3000 rpm for 10 min at room temperature. The resulting supernatants were diluted 50 times with ultrapure distilled water, and portions (50 μl) of the diluted samples were added to tubes containing 100 μl of ammonium molybdate reagent (Biomol Green) for determination of phosphate content by measurement of absorbance at 640 nm. Phosphate solutions of 0 to 40 μM were used as standards. The Na,K-ATPase activity was calculated as the difference in ATPase activity between cells exposed to ouabain and those not, and was expressed as millimoles of ATP hydrolyzed per milligram of protein per hour. The protein concentration of the reaction mixtures was determined by the method of Lowry et al.,[14] with bovine serum albumin as a standard.

Measurement of Pump Function

The pump function of confluent monolayers of corneal endothelial cells was measured with the use of an Ussing chamber basically as described previously.[15,16] The cells cultured on Snapwell inserts were placed in the Ussing chamber EM-CSYS-2 (Physiologic Instruments, San Diego, CA, USA). The endothelial cell surface side was in contact with one chamber and Snapwell membrane side was in contact with another chamber. The chambers were carefully filled with Krebs-Ringer bicarbonate (120.7 mM NaCl, 24 mM $NaHCO_3$, 4.6 mM KCl, 0.5 mM $MgCl_2$, 0.7 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 10 mM glucose bubbled with a mixture of 5% CO_2, 7% O_2, and 88% N_2 to pH 7.4). The chambers were maintained at 37°C by attached heater. The short-circuit current was sensed by narrow polyethylene tubes, positioned close to either side of the Snapwell, filled with 3M KCl and 4% agar gel and connected to silver electrodes. These electrodes were connected to the computer through Ussing system VCC-MC2 (Physiologic Instruments) and iWorx 118 Research Grade Recorder (iWorx Systems, Dover, NH, USA), and the short-circuit current was recorded by Labscribe2 Software for Research (iWorx). After the short-circuit current had reached a steady state, ouabain (final concentration, 1 mM) was added to the chamber, and the short-circuit current was measured again. The pump function attributable to Na,K-ATPase activity was calculated as the difference in short-circuit current measured before and after the addition of ouabain.

Western Blot Analysis of the Na,K-ATPase α_1-Subunit

The culture medium was removed from cell monolayers and then lysed with M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology). The supernatant was collected and total protein in each sample was measured with the use of a BCA Protein Assay Kit (Pierce Biotechnology). Six micrograms of each sample was separated on a 7.5% polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) and then transferred to polyvinylidene (PVDF) membranes. After blocking with 1% normal goat or bovine serum and 0.1% Tween-20 in TBS (TTBS), the membrane was incubated with anti-Na/K-ATPase α_1 antibody (1:5000 dilution with TBS), or anti-Phospho-Na/K-ATPase α_1 (Ser-18) antibody (1:2000 dilution with TBS), or anti β-actin (AC-15) antibody (1:8000 dilution with TBS) overnight at 4°C. Incubation with biotinylated secondary antibodies was followed with the use of Vectastain ABC elite reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. The positive immunoreactions were made visible by an enhanced chemiluminescence ECL Plus Western Blotting Detection Reagent (Amersham Pharmacia Biotech) detection system. A densitometer (ChemiDoc XRS, BioRad, Hercules, CA, USA) was used for quantitation of band intensities.

Statistical Analysis

Data are presented as means ± SD and were compared by Student t-test with the use of Excel 2003 software (Microsoft, Redmond, WA, USA). A p-value of <0.05 was considered statistically significant.

RESULTS

Effect of Dexamethasone on Na,K-ATPase Activity

To determine whether dexamethasone affects Na,K-ATPase activity in corneal endothelial cells, we exposed the cells to 10 μM dexamethasone for various times and then measured such activity. Dexamethasone increased Na,K-ATPase activity in a time-dependent manner, with this effect being significant at 6 hr and maximal at 48 hr (Fig. 1A). The stimulatory effect of dexamethasone on Na,K-ATPase activity was also concentration dependent, being apparent at 1 or 10 μM (Fig. 1B).

To confirm that the stimulatory effect of dexamethasone on Na,K-ATPase activity was mediated by new Na,K-ATPase subunits synthesis, we examined the effects of cycloheximide, a protein synthesis inhibitor. The increase in Na,K-ATPase activity induced by dexamethasone was inhibited by cycloheximide (Fig. 1C). These results thus indicated that the increase in Na,K-ATPase activity induced by dexamethasone is indeed mediated by Na,K-ATPase subunits synthesis.

Effect of Dexamethasone on Pump Function

We next examined whether the activation of dexamethasone affects the pump function of corneal endothelial cells. Tracings of short-circuit current obtained with an Ussing chamber revealed that dexamethasone at 10 μM increased the ouabain-sensitive pump function of the cells compared with that observed for control cells (Fig. 2A). This effect of dexamethasone was time dependent, being significant at 24 hr and maximal at 48 hr (Fig. 2B). The stimulatory effect of dexamethasone on pump function was also concentration dependent,

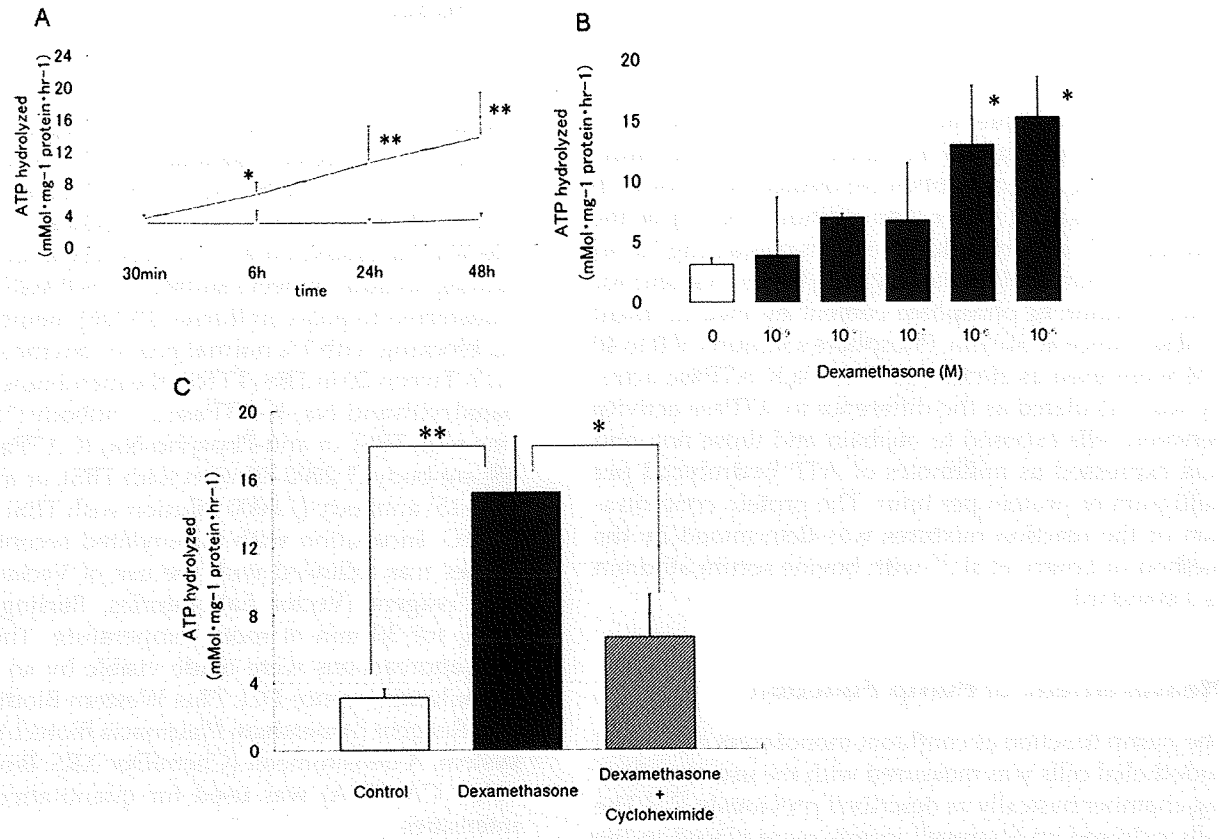

Figure 1. Effect of dexamethasone on Na,K-ATPase activity in cultured mouse corneal endothelial cells. (A) Cells were incubated in the absence (open circles) or presence (open triangles) of 10 μM dexamethasone for the indicated times and then assayed for Na,K-ATPase activity. $*p < 0.05$, $**p < 0.01$ versus the corresponding value for cells incubated without dexamethasone (Student t-test). (B) Cells were incubated with the indicated concentrations of dexamethasone for 48 hr and then assayed for Na,K-ATPase activity. $*p < 0.01$ for the indicated comparisons (Student t-test). (C) Cells were incubated first in the absence or presence of 1 μM cycloheximide for 30 min and then in the additional absence or presence of 10 μM dexamethasone for 48 min, as indicated. $*p < 0.05$, $**p < 0.01$ versus control value (Student t-test). All data are means + SD of values from four replicates from a representative experiment.

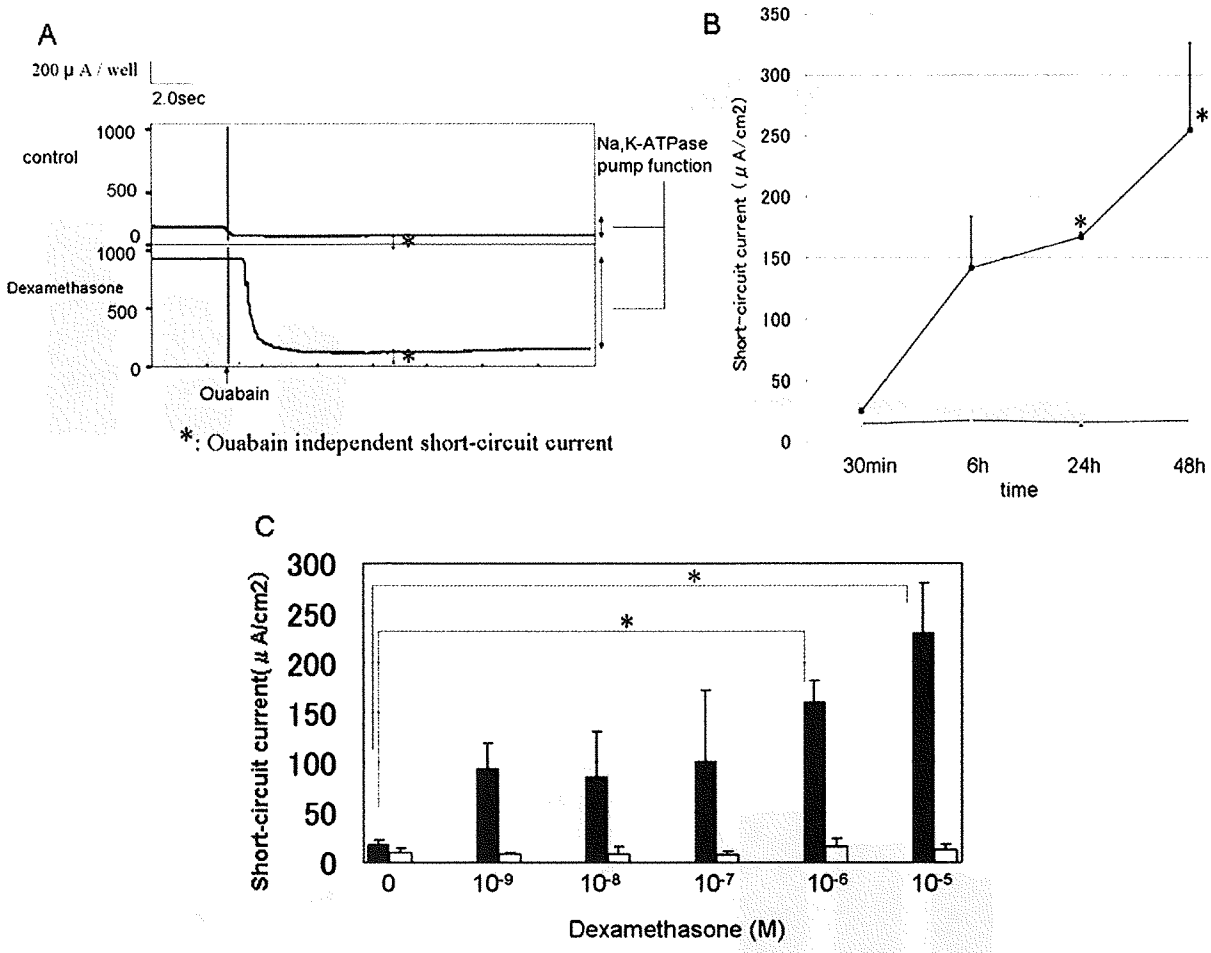

Figure 2. Effect of dexamethasone on the pump function of cultured mouse corneal endothelial cells. (A) Representative tracings of short-circuit current (μA/well) obtained with cell monolayers in an Ussing chamber. Insert well membrane growth area was 4.67 cm^2. The cells were incubated in the absence (upper panel) or presence (lower panel) of 10 μM dexamethasone. Pump function attributable to Na,K-ATPase activity was calculated as the difference in short-circuit currents obtained before and after the addition of ouabain. Ouabain independent short-circuit current (*) represent the influence of other proteins responsible for transport. (B) Cells were incubated in the absence (open circles) or presence (closed squares) of 10 μM dexamethasone for the indicated times and then assayed for pump function (μA/cm^2). *$p < 0.05$ versus the corresponding value for cells incubated without dexamethasone (Student t-test). (C) Pump function (μA/cm^2) attributable to Na,K-ATPase activity was determined 48 hr after incubation of cells in the presence of the indicated concentrations of dexamethasone (black bar). Ouabain independent short-circuit current (white bar) is also presented, which did not change significantly by the indicated concentrations of dexamethasone. Data are means + SD of values from four replicates from a representative experiment. *$p < 0.05$ for the indicated comparisons (Student t-test).

being apparent at 1 or 10 μM (Fig. 2C). Ouabain independent short-circuit current, in that dexamethasone might influence other proteins responsible for transport, did not change significantly (Fig. 2C). These results were similar to the results obtained for Na,K-ATPase activity (Fig. 1A and 1B).

Western Blot Analysis of the Na,K-ATPase α_1-subunit

To determine whether dexamethasone affects Na,K-ATPase expression in corneal endothelial cells, we exposed the cells to 0.1, 1, and 10 μM dexamethasone for 48 hr and then measured the expression of the Na,K-ATPase α_1-subunit and phospho-Na,K-ATPase α_1-subunit by Western blot analysis (Fig. 3A). The phospho-Na,K-ATPase α_1-subunit is considered to be the inactive state of the Na,K-ATPase α_1-subunit. The expression of the Na,K-ATPase α_1-subunit and phospho-Na,K-ATPase α_1-subunit were measured as the ratio of the signal intensity to β-actin. Dexamethasone increased the expression of the Na,K-ATPase α_1-subunit in a concentration-dependent manner (Fig. 3B), whereas it did not

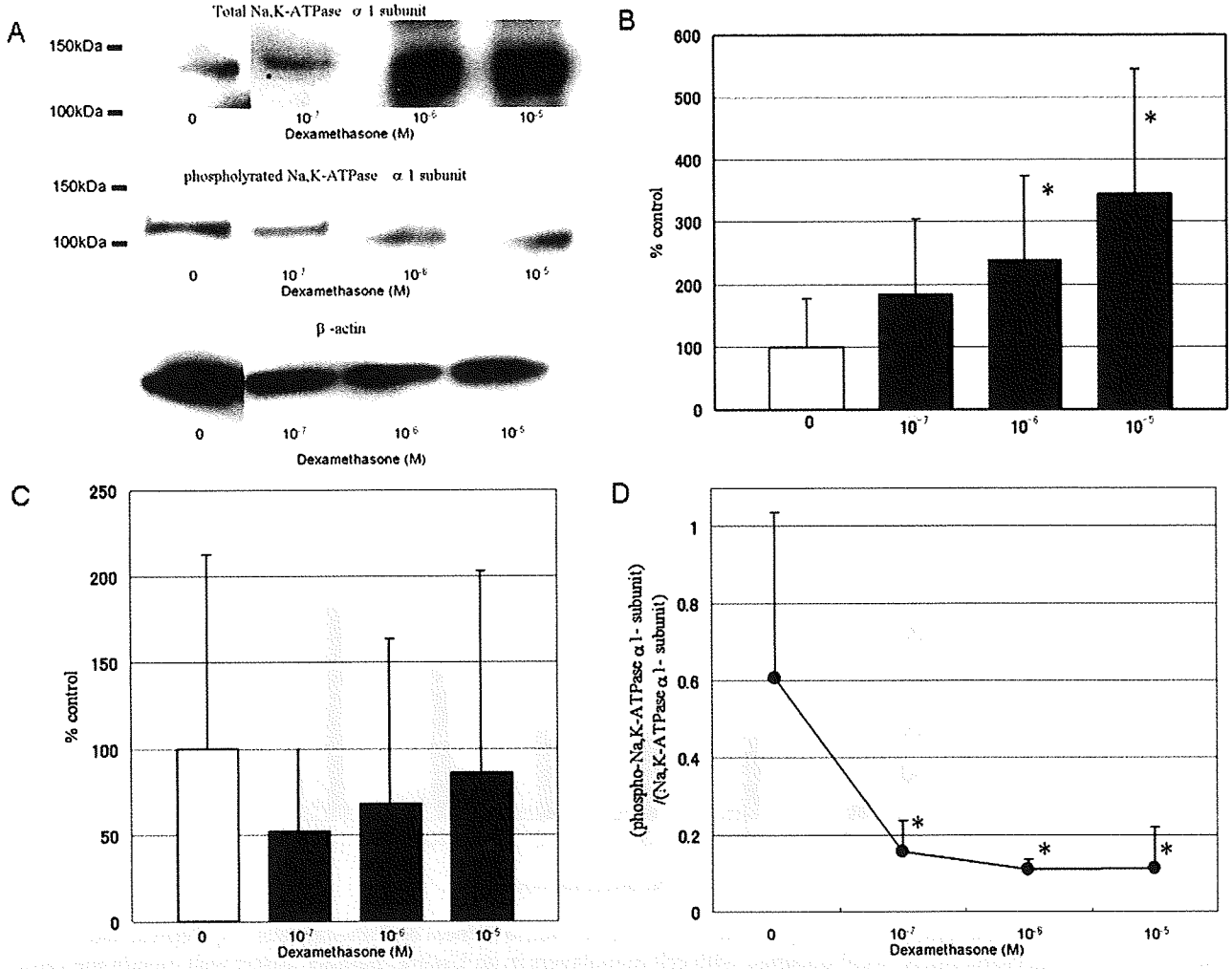

Figure 3. Western blot analysis of Na,K-ATPase α_1-subunit and phospho-Na,K-ATPase α_1-subunit expression. (A) Representative signals of expression. Top: Na,K-ATPase α_1-subunit. Middle: Phospho-Na,K-ATPase α_1-subunit. Bottom: β-actin. For each, the relative intensity of each band to β-actin was measured by the densitometer as the expression of Na,K-ATPase α_1 or phospho-Na,K-ATPase α_1-subunit. (B) Cells were incubated with the indicated concentrations of dexamethasone for 48 hr and then assayed for expression of the Na,K-ATPase α_1-subunit. Data are means + SD from five experiments, expressed as a percentage of control. *$p < 0.05$ for the indicated comparisons (Student t-test). (C) Cells were incubated with the indicated concentrations of dexamethasone for 48 hr and then assayed for expression of the phospho-Na,K-ATPase α_1-subunit. Data are means + SD from five experiments, expressed as a percentage of control. (D) The rate of inactive state of Na,K-ATPase α_1-subunit with the indicated concentrations of dexamethasone. The values represent the ratio of phospho-Na,K-ATPase α_1-subunit expression to Na,K-ATPase α_1-subunit expression. Data are means + SD of values from five experiments. *$p < 0.05$ for the indicated comparisons (Student t-test).

change the expression of the phospho-Na,K-ATPase α_1-subunit (Fig. 3C). Dexamethasone also decreased the ratio of phospho-Na,K-ATPase α_1-subunit expression relative to Na,K-ATPase α_1-subunit expression in a concentration-dependent manner (Fig. 3D). These results indicate that dexamethasone decreases the rate of the inactive state, i.e., increases the rate of the active state of the Na,K-ATPase α_1-subunit.

DISCUSSION

We have shown that dexamethasone results in increases in Na,K-ATPase activity and pump function in cultured corneal endothelial cells. Changes in Na,K-ATPase activity and pump function under the various experimental conditions were well correlated. Our results thus support the notion that Na,K-ATPase activity is an important determinant of the ability of

corneal endothelial cells to maintain the water content of the corneal stroma.[2] The direct stimulation of Na,K-ATPase by steroid hormones has rarely been reported in corneal endothelial cells, even though topical glucocorticoid hormones have been clinically used for the treatment of corneal endothelial disorders. In other tissues, dexamethasone, a glucocorticoid hormone, has been reported to stimulate Na,K-ATPase,[8,17,18] as well as aldosterone, a well-known mineral corticoid hormone stimulating Na,K-ATPase. Lyoussi and Crabbe reported that dexamethasone stimulates Na,K-ATPase more than aldosterone does in amphibian A6 renal distal cells.[17]Recently, Chen et al. demonstrated that dexamethasone has a small but statistically significant effect on the Na,K-ATPase of cultured bovine corneal endothelial cells.[19] Our results support the stimulatory effect of dexamethasone on Na,K-ATPase activity in corneal endothelial cells.

Our results further suggest that the regulation of Na,K-ATPase activity by dexamethasone in corneal endothelial cells is mediated by Na,K-ATPase subunit synthesis. Na,K-ATPase is the largest protein complex in the family of P-type cation pumps, and its minimum functional unit is a heterodimer of the α- and β-subunits.[20] Previous results obtained with amphibian A6 cells suggest that steroid hormones increase Na,K-ATPase α- and β-subunit mRNA abundance.[17] Shinha et al. reported that the administration of dexamethasone to adrenalectomized rats increases the specific activity of Na,K-ATPase in the renal cortex and medulla by increasing the number of enzyme units.[18] Cui and coworkers reported that Na,K-ATPase activity is reduced by cycloheximide in lens epithelial cells, whereas the amounts of Na,K-ATPase protein remain unchanged.[21]They speculated that the pool of Na,K-ATPase protein should be continually replenished by newly synthesized protein to maintain its enzymatic activity.[21] Ewart and Klip also reported that the activation of Na,K-ATPase by steroid hormones appears to be mediated by the synthesis of new α- and β-subunits.[8]

In our study, dexamethasone increased the rate of active state Na,K-ATPase α_1-subunits as well as the total number of Na,K-ATPase α_1-subunits. The anti-phospho-Na/K-ATPase α_1 antibody we used recognizes the Na,K-ATPase α_1-subunit only when phosphorylated at Ser-18. Ser-18 is phosphorylated by protein kinase C,[22–24] and this phosphorylation triggers endocytosis of Na,K-ATPase α_1-subunits and results in inhibition of Na,K-ATPase activity.[25,26] Dexamethasone may prevent Na,K-ATPase α_1-subunits from Ser-18 phosphorylation, and thereby increase the proportion of active-state Na,K-ATPase α_1-subunits.

In conclusion, we have shown that dexamethasone increases Na,K-ATPase activity and pump function in corneal endothelial cells. Furthermore, our results support a model in which Na,K-ATPase activation by dexamethasone in corneal endothelial cells is mediated by Na,K-ATPase subunit synthesis and increased in an enzymatic activity by dephosphorylation of Na,K-ATPase α_1-subunits. Pharmacological manipulation of dexamethasone in corneal endothelial cells is thus a potential therapeutic approach to increasing the pump function of corneal endothelial cells.

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LABORATORY INVESTIGATION

Role of Protein Kinase C in Regulation of Na^+- and K^+-Dependent ATPase Activity and Pump Function in Corneal Endothelial Cells

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Abstract

Purpose: Na^+- and K^+-dependent ATPase (Na,K-ATPase) plays an important role in the pump function of the corneal endothelium. We investigated the possible role of protein kinase C (PKC) in regulation of Na,K-ATPase activity and pump function in corneal endothelial cells.

Methods: Confluent monolayers of mouse corneal endothelial cells were exposed to phorbol 12,13-dibutyrate (PDBu) to induce activation of PKC. ATPase activity of the cells was evaluated by using ammonium molybdate in spectrophotometric measurement of phosphate released from ATP, with Na,K-ATPase activity being defined as the portion of total ATPase activity sensitive to ouabain. Pump function of the cells was measured with a Ussing chamber, with the pump function attributable to Na,K-ATPase activity being defined as the portion of the total short-circuit current sensitive to ouabain.

Results: PDBu (10^{-7} M) increased the Na,K-ATPase activity and pump function of the cultured cells. These effects of PDBu were potentiated by the cyclooxygenase inhibitor indomethacin and the cytochrome P_{450} inhibitor resorufin and were blocked by okadaic acid, an inhibitor of protein phosphatases 1 and 2A.

Conclusions: Our results suggest that PKC bidirectionally regulates Na,K-ATPase activity in mouse corneal endothelial cells: it inhibits Na,K-ATPase activity in a cyclooxygenase- and cytochrome P_{450}-dependent manner, whereas it stimulates such activity by activating protein phosphatases 1 or 2A. **Jpn J Ophthalmol** 2009;53:235–242 © Japanese Ophthalmological Society 2009

Key Words: corneal endothelium, Na,K-ATPase, protein kinase C

Introduction

A single layer of endothelial cells covers the posterior surface of Descemet's membrane in the cornea in a well-arranged mosaic pattern.[1] Corneal hydration is determined primarily by the balance between the penetration of aqueous humor across the corneal endothelium into the stroma and the subsequent pumping of the fluid out from the stroma.[2,3] The accumulation of fluid in the stroma resulting from disturbance of this balance may lead to bullous keratopathy, which is characterized by an edematous cornea with reduced transparency.[4]

Total pumping activity for the removal of fluid from the cornea is determined by the number of endothelial cells and the pump function of each cell. Given that human corneal endothelial cells have a limited proliferative capacity, endothelial dystrophies, ocular trauma, corneal graft rejection, and insults associated with intraocular surgeries may result in corneal endothelial cell loss and permanent damage. Replacement of the corneal endothelium by keratoplasty is currently the only established therapeutic approach to recovery of endothelial cell number. Pseudophakic or aphakic bullous keratopathy, Fuchs' endothelial dystrophy, and failed corneal grafts remain common indications for keratoplasty, accounting for approximately 60% of the total number of such procedures.[5–7]

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Activation of the pump function of the remaining endothelial cells is a potential alternative approach to recovery of the total pumping activity of the cornea, so long as the total number of such cells is within an acceptable range. Indeed, we previously showed that steroid administration appeared to increase endothelial pump function and ameliorated stromal edema in a patient with bullous keratopathy secondary to Sato's refractive surgery.[8] However, therapeutic approaches to the activation of corneal endothelial cells remain to be established.

The Na^+- and K^+-dependent ATPase (Na,K-ATPase) expressed in the basolateral membrane of corneal endothelial cells is primarily responsible for the pump function of the corneal endothelium.[3,9,10] The Na,K-ATPase pump site density in the corneal endothelium has been found to be increased in eyes affected by moderate guttata,[11] whereas it shows an initial increase, a sudden marked decrease, and a subsequent gradual decline in association with the end stage of disease in patients with Fuchs' endothelial dystrophy.[12] These observations indicate that certain conditions can induce a compensatory increase in Na,K-ATPase pump site density and, presumably, in endothelial pump function. They thus also suggest the existence of a regulatory mechanism (or mechanisms) for control of total Na,K-ATPase activity in the corneal endothelium.

Various hormones and neuromediators, including aldosterone, thyroid hormone, insulin, and catecholamines, have been shown to regulate Na,K-ATPase in various tissues.[13] These agents activate several signaling pathways, including those mediated by protein kinases such as protein kinase C (PKC). PKC actually comprises a family of at least ten related serine-threonine kinases that are activated by phorbol esters.[14] A direct effect of PKC on Na,K-ATPase activity has been demonstrated in various tissues.[13,14] A phorbol ester-induced increase in rabbit corneal endothelial permeability and corneal swelling mediated by activation of PKC have been described, but PKC has also been shown to induce changes in endothelial cell structure and an acute increase in cell junctional permeability.[15] The role of PKC in regulating Na,K-ATPase activity in corneal endothelial cells, however, remains unclear.

In the present study, we investigated the role of PKC in the control of Na,K-ATPase in corneal endothelial cells. We examined the effects of PKC activation on the enzymatic activity and pump function of Na,K-ATPase in cultured mouse corneal endothelial cells. In addition, we examined the mechanisms by which PKC might affect Na,K-ATPase activity by using inhibitors of cyclooxygenase, cytochrome P_{450}, and protein phosphatases.

Methods

Chemicals

Ammonium molybdate reagent (Biomol Green), phosphate standards and 4α-phorbol 12,13-dibutyrate (4α-PDBu) were obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Phorbol 12,13-dibutyrate (PDBu), staurosporine, ouabain, indomethacin, resorufin, okadaic acid, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Water-insoluble compounds were dissolved in a minimal volume of methanol or dimethyl sulfoxide, with equal amounts of these solvents being added to control solutions; the final concentration of methanol or dimethyl sulfoxide in incubations was <0.3% and was found to have no effect on ATP hydrolysis or short-circuit currents (data not shown).

Cell Culture

A simian virus 40-transformed mouse corneal endothelial cell line (C3H) was kindly provided by J. W. Sreilein (Harvard Medical School). The cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were passaged at a split ratio of 1:2 to 1:4, becoming confluent in 4 to 7 days. Experiments examining PKC activity or Na,K-ATPase activity were performed with cells cultured in 24-well culture plates (Nunc, Roskilde, Denmark). Ussing chamber experiments were performed with confluent monolayers of cells cultured in six-well polycarbonate Snapwell inserts (Corning, Acton, MA, USA) with a membrane pore size of 0.4 μm. All experiments were performed with cell monolayers within 1 day of the cells' reaching confluence and with the cells maintained in the culture incubator at 37°C.

Measurement of PKC Activity

After removal of the culture medium, cells were washed with phosphate-buffered saline and lysed in 1 ml of a solution containing 20 mM MOPS (pH 7.4X), 50 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium vanadate, 5 mM EGTA, 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), and aprotinin (10 μg/ml). The lysates were centrifuged at 2.6×10^4 *g* for 15 min at 4°C, and the resulting supernatants were assayed for the kinase activity of PKC with a PKC Kinase Activity assay kit (Assay Designs, Ann Arbor, MI, USA), which is based on a solid-phase enzyme-linked immunosorbent assay that recognizes the phosphorylated form of the substrate. PKC activity was expressed as nanograms of phosphorylated substrate formed per milligram of protein. The protein concentration of lysate supernatants was assayed by the method of Lowry et al.[16] with bovine serum albumin as a standard.

Measurement of Na,K-ATPase Activity

The culture medium was removed from the cell monolayers, ultrapure distilled water (150 μl) was added to each

well, and the culture plate was then placed in liquid nitrogen for 10 s before the addition to each well at room temperature of 150 µl of a solution containing 80 mM histidine, 20 mM KCl, 6 mM $MgCl_2$, 2 mM EGTA, alamethicin (2 µg/ml), 30 µM digitonin, and 200 mM NaCl at pH 7.4.[17] To duplicate wells, 10 µl of 30 mM ouabain or vehicle was added, and the plate was incubated for 30 min at 37°C. After the further addition of 10 µl of 300 mM ATP (final concentration, 10 mM), the reaction mixtures were incubated for an additional 30 min at 37°C. The ATP hydrolysis reaction was terminated by the addition of 75 µl of 50% trichloroacetic acid to each well. The contents of each well were then centrifuged at $1.4 \times 10^5 g$ for 10 min at room temperature. The resulting supernatants were diluted 50 times with ultrapure distilled water, and portions (50 µl) of the diluted samples were added to tubes containing 100 µl of ammonium molybdate reagent (Biomol Green) for determination of phosphate content by measurement of absorbance at 640 nm. Phosphate solutions of 0–40 µM were used as standards. The Na,K-ATPase activity was calculated as the difference in ATPase activity between cells exposed to ouabain and those not, and was expressed as millimoles of ATP hydrolyzed per milligram of protein per hour. The protein concentration of the reaction mixtures was determined by the method of Lowry et al.[16] with bovine serum albumin as a standard.

Measurement of Pump Function

The pump function of the confluent monolayers of corneal endothelial cells was measured in an Ussing chamber, basically as described previously.[18,19] The cells cultured on Snapwell inserts were placed in the Ussing chamber and incubated in Krebs-Ringer bicarbonate (120.7 mM NaCl, 24 mM $NaHCO_3$, 4.6 mM KCl, 0.5 mM $MgCl_2$, 0.7 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 10 mM glucose bubbled with a mixture of 5% CO_2, 7% O_2, and 88% N_2 to pH 7.4). After the short-circuit current had reached a steady state, ouabain (final concentration, 1 mM) was added to the chamber and the short-circuit current was measured again. The pump function attributable to Na,K-ATPase activity was calculated as the difference in short-circuit current measured before and after the addition of ouabain.

Statistical Analysis

Data are presented as means ± SD and were compared by Student's *t* test by using Excel 2003 software (Microsoft, Redmond, WA, USA). A *P* value of <0.05 was considered statistically significant.

Results

Activation of PKC by PDBu in Corneal Endothelial Cells

To examine whether PDBu activates PKC in cultured mouse corneal endothelial cells, we measured PKC activity in cell extracts after treatment of the cells with various concentrations of the phorbol ester for 30 min. PDBu indeed increased PKC activity in a concentration-dependent manner, with this effect being significant and maximal at concentrations of 10^{-7} to 10^{-5} M (Fig. 1).

Effect of PDBu on Na,K-ATPase Activity

To determine whether activation of PKC affected Na,K-ATPase activity in corneal endothelial cells, we exposed the cells to various concentrations of PDBu and then measured such activity. The stimulatory effect of PDBu on Na,K-ATPase activity was apparent at 10^{-7} M but not at the higher concentrations of 10^{-6} and 10^{-5} M (Fig. 2A).

To confirm that the stimulatory effect of PDBu on Na,K-ATPase activity was mediated by PKC, we examined the effects both of 4α-PDBu, a structural analog of PDBu that does not activate PKC, and of the PKC inhibitor staurosporine. The increase in Na,K-ATPase activity induced by PDBu was not mimicked by 4α-PDBu and was inhibited by staurosporine (Fig. 2B). These results indicated that the increase in Na,K-ATPase activity induced by PDBu at a concentration of 10^{-7} M was indeed mediated by PKC, but that higher concentrations of PDBu did not affect Na,K-ATPase activity even though they activated PKC.

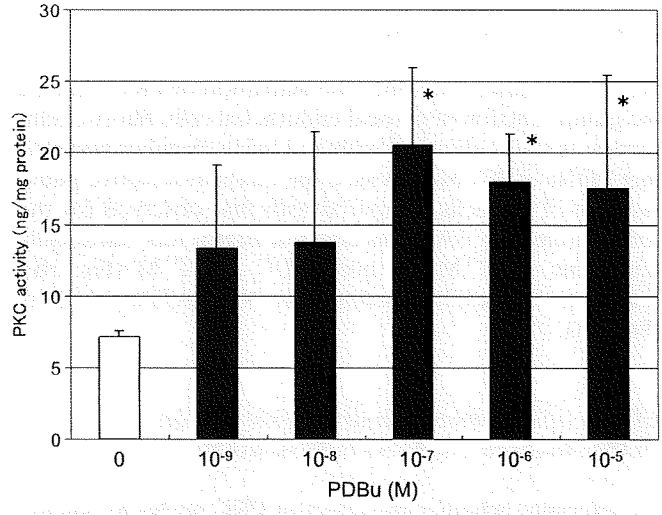

Figure 1. Effect of phorbol 12,13-dibutyrate (*PDBu*) concentration on protein kinase C (*PKC*) activity in cultured mouse corneal endothelial cells. Cells were incubated with the indicated concentrations of PDBu for 30 min, after which the activity of PKC was measured in cell extracts. Data are means + SD of values from four replicates from a representative experiment. $*P < 0.05$ versus the value for cells incubated without PDBu (Student's *t* test).

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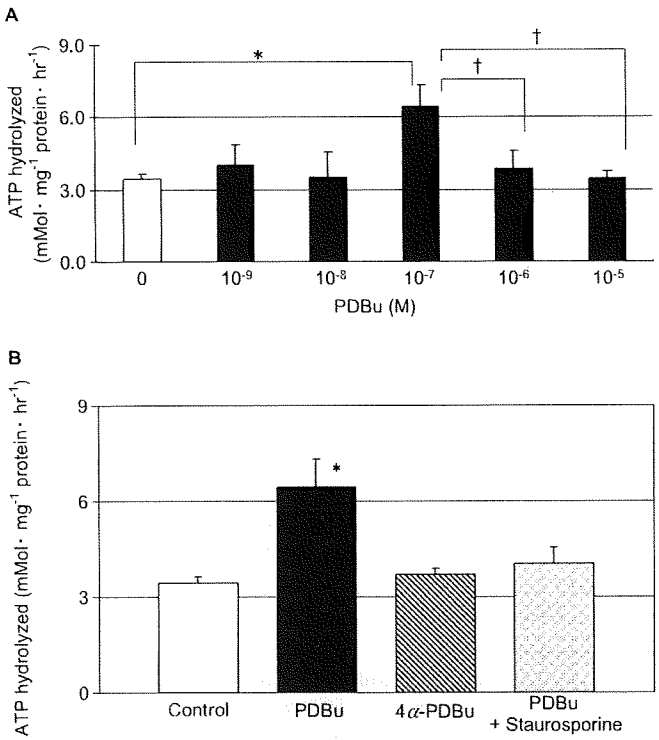

Figure 2A, B. Effect of PDBu on Na^+- and K^+-dependent ATPase (Na,K-ATPase) activity in cultured mouse corneal endothelial cells. **A** Cells were incubated with the indicated concentrations of PDBu for 30 min and then assayed for Na,K-ATPase activity. $*P < 0.001$, $\dagger P < 0.01$ for the indicated comparisons (Student's t test). **B** Cells were incubated first in the absence or presence of 10^{-5} M staurosporine for 30 min and then in the additional absence or presence of 10^{-7} M PDBu or 10^{-7} M 4α-PDBu for 30 min, as indicated. $*P < 0.001$ versus the control value (Student's t test). All data are means + SD of values from four replicates from a representative experiment.

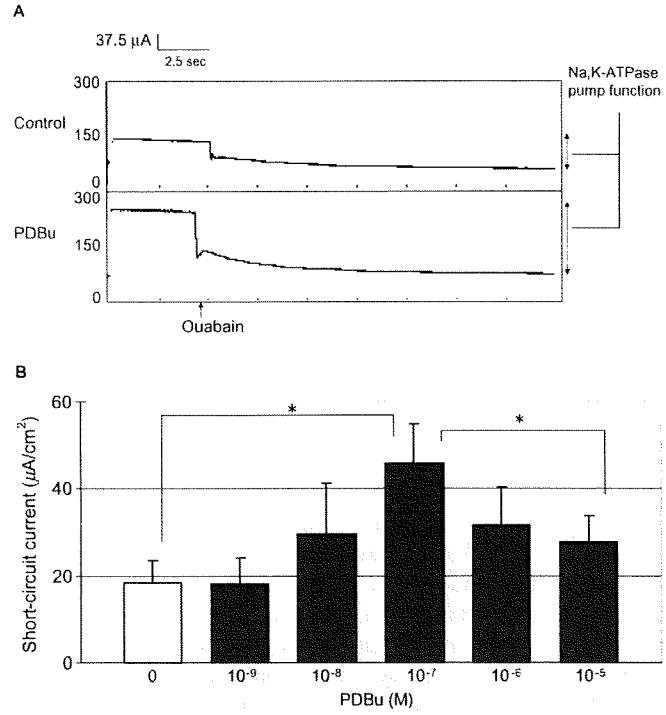

Figure 3A, B. Effect of PDBu on the pump function of cultured mouse corneal endothelial cells. **A** Representative short-circuit current tracings obtained with cell monolayers in an Ussing chamber. The cells were incubated in the absence (*upper panel*) or presence (*lower panel*) of 10^{-7} M PDBu. Pump function attributable to Na,K-ATPase activity was calculated as the difference in short-circuit currents obtained before and after the addition of ouabain. **B** Pump function attributable to Na,K-ATPase activity was determined 30 min after incubation of cells in the presence of the indicated concentrations of PDBu. Data are means + SD of values from four replicates from a representative experiment. $*P < 0.05$ for the indicated comparisons (Student's t test).

Effect of PDBu on Pump Function

We next examined whether the activation of PKC affected the pump function of corneal endothelial cells. Short-circuit current tracings obtained with an Ussing chamber revealed that PDBu at 10^{-7} M increased the ouabain-sensitive pump function of the cells compared with that observed for the control cells (Fig. 3A). This effect of PDBu was statistically significant at 10^{-7} M but not at 10^{-6} or 10^{-5} M (Fig. 3B), similar to the results obtained for Na,K-ATPase activity (Fig. 2A).

Effects of Indomethacin and Resorufin on PDBu-Induced Na,K-ATPase Activity

To determine whether the effect of PKC on Na,K-ATPase activity was mediated indirectly through effects on cyclooxygenase or cytochrome P_{450}, we examined whether the cyclooxygenase inhibitor indomethacin or the cytochrome P_{450} inhibitor resorufin affected Na,K-ATPase activity in cells exposed to various concentrations of PDBu. The stimulatory effect of PDBu alone on Na,K-ATPase activity was again apparent at 10^{-7} M but not at 10^{-6} or 10^{-5} M. The additional presence of 10^{-6} M indomethacin resulted in significant increases in Na,K-ATPase activity compared with the control at 10^{-7} M and 10^{-6} M PDBu, and with that in the presence of PDBu alone at 10^{-6} M PDBu (Fig. 4A). The additional presence of 10^{-6} M resorufin resulted in significant increases in Na,K-ATPase activity compared with the control at 10^{-7} M, 10^{-6} M, and 10^{-5} M PDBu, and with that in the presence of PDBu alone at 10^{-5} M PDBu (Fig. 4B). The additional presence of both 10^{-6} M indomethacin and 10^{-6} M resorufin resulted in significant increases in Na,K-ATPase activity compared with the control at 10^{-7} M, 10^{-6} M, and 10^{-5} M PDBu in a dose-dependent manner, and with that in the presence of PDBu alone, with the most pronounced effects at 10^{-6} M and 10^{-5} μM PDBu (Fig. 4C). Neither indomethacin nor resorufin, nor the combination of these agents, affected Na,K-ATPase activity in the absence of PDBu. These results suggest that the higher concentrations (10^{-6} and 10^{-5} M) of PDBu had bidirectional (both stimulatory and inhibitory) effects on Na,K-ATPase activity. The inhibitory effect appeared to be mediated at least in part through cyclooxygenase and cytochrome P_{450}.

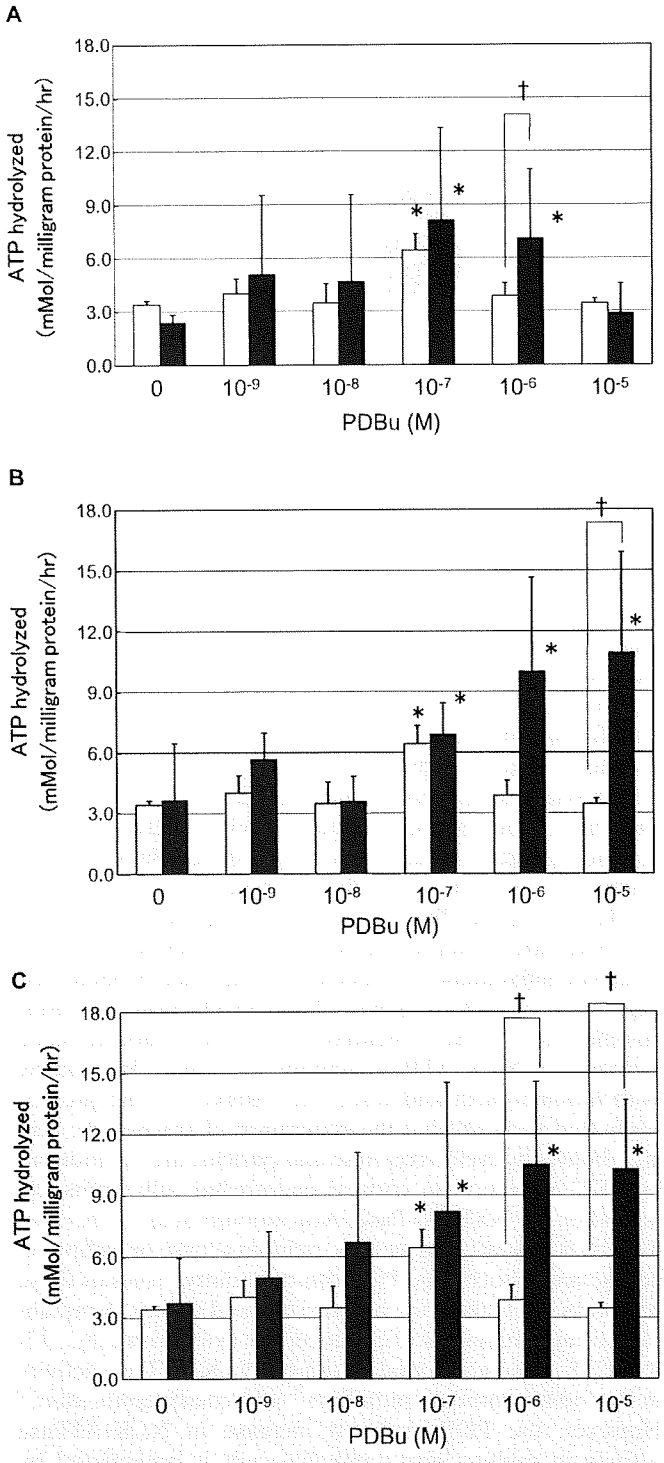

Figure 4A–C. Effects of indomethacin and resorufin on PDBu-induced Na,K-ATPase activity in cultured mouse corneal endothelial cells. Cells were incubated first for 30 min in the absence (*white bars*) or presence (*black bars*) of 10^{-6} M indomethacin (**A**), 10^{-6} M resorufin (**B**), or both 10^{-6} M indomethacin and 10^{-6} M resorufin (**C**) and then for an additional 30 min in the additional presence of the indicated concentrations of PDBu. The activity of Na,K-ATPase in the cells was then measured. $*P < 0.05$ versus the control value (*white bar* at 0 μM PDBu). $\dagger P < 0.05$ for the indicated comparisons (Student's *t* test). All data are means + SD of values from four replicates from a representative experiment.

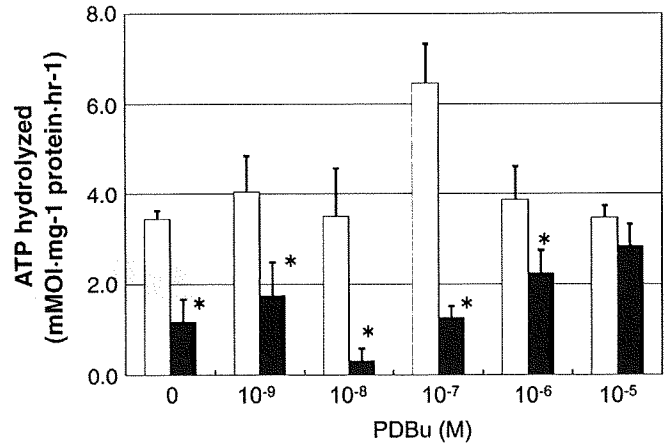

Figure 5. Effect of okadaic acid on PDBu-induced Na,K-ATPase activity in cultured mouse corneal endothelial cells. Cells were incubated first for 30 min in the absence (*white bars*) or presence (*black bars*) of 10^{-6} M okadaic acid and then for an additional 30 min in the additional presence of the indicated concentrations of PDBu before measurement of Na,K-ATPase activity. $*P < 0.05$ versus PDBu alone (*white bar*) (Student's *t* test). All data are means + SD of values from four replicates from a representative experiment.

Effect of Okadaic Acid on PDBu-Induced Na,K-ATPase Activity

We next examined whether okadaic acid, an inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), might affect the increase in Na,K-ATPase activity induced by PKC. The activity of Na,K-ATPase at various concentrations of PDBu was significantly reduced in the additional presence of 10^{-6} M okadaic acid, with the increase in Na,K-ATPase activity induced by PDBu at 10^{-7} M being inhibited by this agent (Fig. 5). These results suggest that the activity of PP1 or PP2A is essential to the maintenance of Na,K-ATPase activity.

Effects of Indomethacin, Resorufin, and Okadaic Acid on Pump Function

Finally, we examined the effects of indomethacin, resorufin, and okadaic acid on the PKC-induced increase in pump function in corneal endothelial cells. The increase in ouabain-sensitive pump function induced by 10^{-7} M PDBu was potentiated by indomethacin and resorufin in an approximately additive manner and was prevented by okadaic acid (Fig. 6). These results are thus similar to those obtained for the effects of these agents on Na,K-ATPase activity (Figs. 4 and 5).

Discussion

We showed that the activation of PKC resulted in increases in Na,K-ATPase activity and pump function in cultured corneal endothelial cells, though the stimulatory effect of

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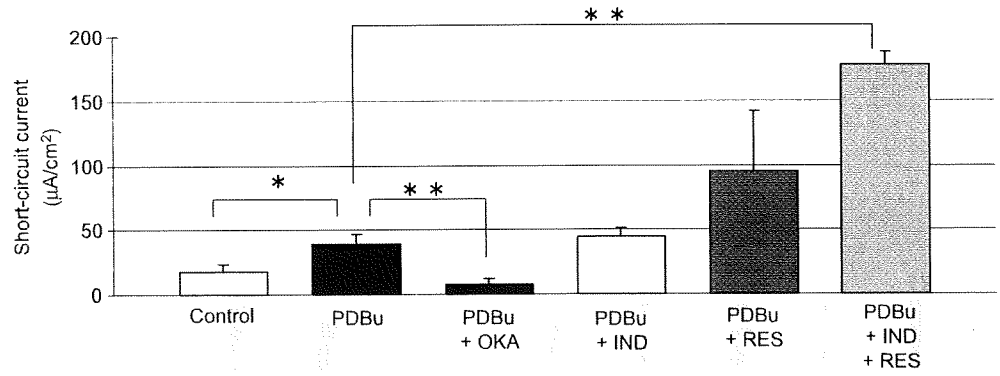

Figure 6. Effects of okadaic acid, indomethacin, and resorufin on the pump function of cultured mouse corneal endothelial cells. Cells were incubated for 30 min in the absence or presence of 10^{-7} M okadaic acid (*OKA*), 10^{-6} M indomethacin (*IND*), or 10^{-6} M resorufin (*RES*), and then pump function was determined during incubation for an additional 30 min in the additional absence or presence of 10^{-7} M PDBu, as indicated. Data are means + SD of values from four replicates from a representative experiment. $^{*}P < 0.05$, $^{**}P < 0.01$ for the indicated comparisons (Student's *t* test).

PDBu on Na,K-ATPase activity was apparent at 10^{-7} M but not at the higher concentrations of 10^{-6} and 10^{-5} M PDBu, resulting in a bell-shaped activation curve. Changes in Na,K-ATPase activity and pump function under the various experimental conditions were well correlated. Our results thus support the notion that Na,K-ATPase activity is an important determinant of the ability of corneal endothelial cells to maintain the water content of the corneal stroma.[3,4,9,10] They further suggest that the regulation of Na,K-ATPase activity by PKC in corneal endothelial cells is mediated indirectly through activation of inhibitory pathways mediated by cyclooxygenase and cytochrome P_{450} as well as a stimulatory pathway.

Bidirectional regulation of Na,K-ATPase activity by PKC has been described previously in other cell types and tissues.[13] PDBu has been shown to have positive and negative effects on Na,K-ATPase activity in the renal cortex.[14] In addition, activation of PKC by PDBu resulted in stimulation of Na,K-ATPase activity in rat proximal convoluted tubules under oxygenated conditions but in inhibition of such activity under hypoxic conditions.[20] With regard to ocular tissues, bidirectional regulation of Na,K-ATPase activity by PKC has also been demonstrated in the ciliary epithelium.[17]

We found that the increases in Na,K-ATPase activity and pump function induced by PDBu in corneal endothelial cells were potentiated by the cyclooxygenase inhibitor indomethacin and the cytochrome P_{450} inhibitor resorufin. Indomethacin seemed to release the restriction of Na,K-ATPase activation at the concentration of 10^{-6} M PDBu, and resorufin seemed to release the restriction at the concentration of 10^{-5} M PDBu. In the presence of indomethacin and resorufin, both restrictions were released, making it appear that PDBu increased Na,K-ATPase activity in a dose-dependent manner. These results suggest that the higher concentrations (10^{-6} and 10^{-5} M) of PDBu have bidirectional (both stimulatory and inhibitory) effects on Na,K-ATPase activity and that the inhibitory effect is mediated through cyclooxygenase and cytochrome P_{450}. The inhibitory effects may cause the bell-shaped Na,K-ATPase activation curve by PDBu. Both the release of arachidonic acid from phospholipids catalyzed by phospholipase A_2 and the subsequent metabolism of arachidonic acid are promoted by the phorbol ester-induced activation of PKC in various cell types.[21–23] Metabolism of arachidonic acid generates a wide range of products, including prostaglandin E_2, prostaglandin $F_{2\alpha}$, 5-hydroxyeicosatetraenoic acid (5-HETE), and 12-HETE,[24] and these metabolites influence cell function.[20] Such arachidonic acid metabolites thus inhibit Na,K-ATPase activity[25–27] as well as promote inflammation.[28–30] Topical application of the PKC activator 12-*O*-tetradecanoylphorbol 13-acetate to mouse skin was previously shown to induce a marked inflammatory response accompanied by increased expression of cyclooxygenase-2 and production of prostaglandin E_2.[29,30] The potentiation of the PDBu-induced increases in Na,K-ATPase activity and pump function by both indomethacin and resorufin observed in the present study thus suggests that the generation of arachidonic acid metabolites by cyclooxygenase and cytochrome P_{450} induced by PKC activation in corneal endothelial cells limits the activation of Na,K-ATPase. Arachidonic acid is metabolized by pathways mediated by cyclooxygenase or by lipoxygenase and cytochrome P_{450}, with the relative roles of these pathways likely differing among cells and tissues. A metabolite of arachidonic acid produced by cytochrome P_{450}, 12-*R*-HETE, has been found to inhibit Na,K-ATPase activity in the rabbit corneal epithelium[31] and ciliary epithelium.[32] However, the PDBu-induced increase in Na,K-ATPase activity in rabbit ciliary epithelial cells is potentiated by indomethacin but unaffected by resorufin or by an inhibitor of lipoxygenase.[20]

We found that both Na,K-ATPase activity and the endothelial pump function were suppressed by okadaic acid even in the presence of PDBu. These results suggest that PP1 or PP2A is essential for the activity of Na,K-ATPase in corneal endothelial cells. Several previous studies have shown that PKC stimulates Na,K-ATPase activity via protein phosphatases.[33,34] Deachapunya and associates[33] have reported that

phosphoinositide 3-kinase and PKC are key signal pathways in activation of PP1 and PP2A and in the consequent dephosphorylation of the α subunit of Na,K-ATPase, which in turn results in Na,K-ATPase activation. Layne and associates[34] have suggested that the activation of PKC increases the activity of PP1 in human pigmented ciliary epithelial cells. Although the mechanism by which PKC stimulates Na,K-ATPase activity is unclear in our study, PP1 and/or PP2A may play a significant role in the stimulatory pathway.

In this study, we used staurosporine as the PKC inhibitor, indomethacin as the cyclooxygenase inhibitor, resorufin as the cytochrome P_{450} inhibitor, and okadaic acid as the PP1 and PP2A inhibitor. It is unclear whether these chemicals may affect the cells through other unknown mechanisms, and this is a limitation of this study. Further investigation is needed to explore the mechanism of stimulation of Na,K-ATPase activity by PKC.

In conclusion, we showed that PDBu increases Na,K-ATPase activity and pump function in corneal endothelial cells. Furthermore, our results support a model in which PKC exerts bidirectional control of Na,K-ATPase activity in corneal endothelial cells. It inhibits Na,K-ATPase activity in a cyclooxygenase- or cytochrome P_{450}-dependent manner, whereas it increases such activity, possibly by activating protein phosphatases. Pharmacological manipulation of PKC in corneal endothelial cells is thus a potential new therapeutic approach to increasing the pump function of corneal endothelial cells.

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Turnover rate of tear-film lipid layer determined by fluorophotometry

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ABSTRACT

Aim: This study was performed to independently assess the turnover rates of aqueous and lipid layers of the tear film.

Methods: Two fluorescent dyes, fluorescein sodium and 5-dodecanoylaminofluorescein (DAF), which is a free-fatty-acid conjugate of fluorescein, were applied to the right eye of 12 healthy volunteers. Fluorescent intensity of the precorneal tear film was measured at the central cornea every minute for 10 min for fluorescein sodium, and every 5 min for 50 min for DAF. The turnover rate was calculated by plotting fluorescent intensity against time in a semilog plot and expressed as %/min.

Results: Turnover rates of fluorescein sodium and DAF were 10.3 (SD 3.7)%/min and 0.93 (0.36)%/min, respectively. The turnover rate of DAF was significantly lower than that of fluorescein sodium ($p<0.05$, Mann–Whitney test). The turnover rate of DAF positively correlated with that of fluorescein sodium ($r = 0.93$, $p<0.05$).

Conclusion: Our results indicate that the turnover of lipids in tears is much slower than the aqueous flow of tears, and that this lipid turnover is associated with the aqueous flow of tears in healthy adults.

The precorneal tear film has traditionally been described as consisting of an outer lipid layer, a middle aqueous layer and an inner mucus layer. Although this remains valid, some modifications have been proposed.[1–5] In the current model of the tear film, the aqueous-mucin layer is covered by two thin layers of lipids. Polar lipids such as phospholipids lie adjacent to the aqueous-mucin layer, and non-polar lipids such as cholesterol and wax ester are present at the tear–air interface. In addition, tears contain proteins that possess lipid-binding properties, such as tear lipocalin.[2–6] Although lipids in tears are primarily located in the tear-film lipid layer, some lipids are presumably bound by lipocalin in the aqueous layer. Tear lipocalin is thought to have an important role in stabilising the tear-film lipid layer by transferring lipids to it from the aqueous layer.[4–6]

Despite comprising a very small proportion of the overall tear-film thickness, the lipid layer is important for retarding evaporation and maintaining tear-film stability.[3 5] Where the lipid layer is absent or where the integrity of the lipid layer is compromised, the evaporation rate of tears increases, accompanied by tear-film instability.[7 8] To assess the lipid layer of tears, several techniques have been developed, including observation of lipid layer characteristics by interferometric methods,[9–11] quantitative measurement of meibomian lipid on the lid margin by meibometry[12 13] and measurement of evaporation from the ocular surface.[14–16] Of these, observation of lipid layer characteristics by interferometric methods has been well established.[9–11 17] In various pathological conditions, such as meibomian gland dysfunction, the appearance of the lipid layer can change. Lipid layer thickness, measured by interferometry, has been reported to correlate with tear-film evaporation, tear-film breakup time, and clinical symptoms.[8 18] We have previously reported that the concentration of lipocalin in tears from patients with meibomian gland dysfunction was significantly lower than in normal controls.[17] Thus, lipids in tears, both in the lipid layer and in the aqueous layer held by lipocalin, are important when considering the pathophysiology of evaporative dry eye, such as meibomian gland dysfunction. Until now, however, there has been no information about the flow rate of tear-film lipid layer.

Aqueous tear flow is determined by several aspects of tear dynamics including tear production, tear volume, tear evaporation and tear outflow.[20] Tear flow can be assessed by introducing a dye or radioactive substance into the conjunctival sac and measuring the decay in concentration over a certain period. Since the report of Mishima *et al*,[21] fluorophotometric measurement using fluorescein sodium as a tracer has been the gold standard to quantify tear flow.[16 20] The elimination rate of fluorescein sodium essentially represents the bulk aqueous flow because the dye is hydrophilic; however, the turnover of a certain tear component may not parallel the bulk aqueous flow. For example, we recently reported differences between the bulk aqueous flow of hyaluronic acid and the turnover of hyaluronic acid, suggesting that hyaluronic acid remains on the ocular surface independent of the bulk aqueous flow.[22] Accordingly, we hypothesised that the flow rate of the tear lipid layer might be different from that of aqueous tear layer.

In this study, we tested this hypothesis using fluorescein sodium and a free-fatty-acid conjugate of fluorescein. Fluorescein was used to assess the aqueous flow, and the conjugated dye was used as a tracer to determine the flow rate of the tear lipid layer.

METHODS

Fluorescent dye and fluorophotometer

5-Dodecanoylaminofluorescein (DAF; Molecular Probes, Eugene, Oregon) is a lipophilic and water-insoluble free-fatty-acid conjugate of fluorescein. This dye has the longest-wavelength absorption maximum at 495 nm, and an emission spectrum that peaks at 518 nm. A DAF emulsion (50 mg/ml) was prepared in sterile 0.067 M phosphate-buffered saline (PBS), pH 7.4, with 1% Tween 80

(Sigma-Aldrich, St Louis, Missouri). Fluorescein sodium (Sigma-Aldrich) was dissolved in sterile 0.067 M PBS and used as a tracer of the tear aqueous layer. No signs of inflammation or damage were detected either immediately or after 24 h by instilling five drops of 5% DAF emulsion at 10 min intervals into four rabbits' eyes. Instillation of one drop of 5% DAF emulsion into the eyes of four subjects caused no discomfort, and no staining or adverse effects were detected by a slit-lamp examination. To test the effect of DAF emulsion on tear-film stability, we measured tear-film break-up time (BUT) after instilling a 1 µl of DAF emulsion (50 mg/ml). The DAF-BUT was 21.6 (10.4) s (n = 20), which was longer than fluorescein BUT (13.1 (3.6) s) measured by instilling 1 µl of fluorescein sodium solution (50 µg/ml) on a different day. From these preliminary experiments, a DAF emulsion was considered to have no apparent adverse effects on the ocular surface or the tear film.

A modified Bligh and Dyer procedure was performed to test the nature of these dyes. Ten microlitres of 5% DAF emulsion and 0.5% fluorescein sodium solution were placed in a test tube with 1 ml of a 2:1 chloroform:methanol solvent (Wako, Osaka, Japan). After adding 0.4 ml of water, the tubes were vortexed for 30 s. The solvent formed two layers—an upper aqueous layer and a lower lipid layer. As expected, the fluorescent yellowish colour of DAF was seen in the lower lipid layer, whereas the dye colour of fluorescein sodium was seen in the upper aqueous layer (fig 1). Spectrophotometric measurements (495 nm) revealed that 99% of DAF was located in the lower lipid layer, and 97% of fluorescein sodium was in the upper aqueous layer.

A commercial slit-lamp fluorophotometer (Anterior Fluorometer FL-500, Kowa, Tokyo) was used to quantify fluorescence intensity. The illuminating light was focused as a 2 mm diameter circle on the surface of the cornea. The emitted light passed through a band interference filter centred on 565 nm (half bandwidth 25 nm) and was directed to a photomultiplier tube with the band interference filter centred on a wavelength of 490 nm (half bandwidth 30 nm).

DAF emulsion (50 mg/ml) was diluted in PBS to produce standards ranging from 0.01 to 50 mg/ml for calibration. Fluorescein sodium solution (5 mg/ml) was diluted to produce standards ranging from 0.1 to 50 µg/ml in the same fashion. A cuvette was constructed by gluing together two microscopic slides and two cover glasses. The cover glasses were sandwiched by the two slides to provide a 12–15 µm thick space for the fluid layer. A fresh cuvette was used for each solution. The standards (10 µl) were added to the cuvettes, and fluorescence intensity was measured by the slit-lamp fluorophotometer. The interaction of DAF or fluorescein sodium with proteins was also tested by diluting the standards in PBS containing 10% fetal bovine serum (FBS).

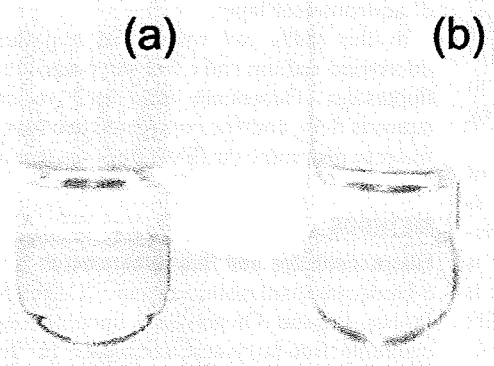

Figure 1 Fluorescein sodium (A) and 5-dodecanoylaminofluorescein (DAF) (B) after a modified Bligh and Dyer procedure. The fluorescent yellowish colour of fluorescein sodium was seen in the upper aqueous layer, whereas the dye colour of DAF was seen in the lower lipid layer.

Measurement of turnover rate

Twelve healthy volunteers (six males and six females) aged 21 to 47 years (mean 32.6 (SD 8.2) years), who had no history of eye disease except for refractive errors, were enrolled in the study. One of the authors (SH) performed a routine ocular examination on all subjects, followed by an examination of the ocular surface, including Schirmer testing and measurement of tear-film break-up time (BUT). For vital staining, 2 µl of a saline solution containing 1% fluorescein was used. All subjects had more than 5 mm of Schirmer strip wetting, more than 5 s in tear-film BUT, and no apparent fluorescein staining of cornea and conjunctiva. The Marx lines, which run along the eyelid margin determined by fluorescein staining, were normal in all subjects.[23] The guidelines of the World Medical Association Declaration of Helsinki were followed. The subjects received a full explanation of the procedures and provided their informed consent for participation prior to the experiment. The protocol was approved by the institutional review board of National Tokyo Medical Center (R-07-011: Assessment of layer-by-layer tear dynamics by fluorophotometry), and all subjects provided written informed consent.

The subjects were seated in front of the fluorophotometer. The instrument was focused on the central cornea, and background fluorescence intensity was measured. A volume of 1 µl of DAF emulsion (50 mg/ml) or fluorescein sodium solution (50 µg/ml) was applied to the right eye using an Eppendorf micropipette without making contact with the ocular surface. The subjects were instructed to blink several times to ensure mixing of the dye. Fluorescence intensity of the precorneal tear film was measured at the central cornea. When fluorescein sodium solution was instilled into the eye, fluorescence intensity was measured every minute for 10 min. When DAF emulsion was instilled, measurements were repeated every 5 min for 50 min because of a slower decay of intensity. Measurements of fluorescein sodium and DAF were done on different days. Measurements of DAF were repeated on three different days in three subjects to evaluate the repeatability of the test.

The turnover rate was determined by plotting fluorescence intensity against time in a semilog plot, $F = F_0\exp(-kt)$, where F = fluorescence intensity at time (t), F_0 = fluorescence intensity at time zero, k = turnover rate and t = time (min). The turnover rate was calculated for all tests and expressed as %/min. The regression fit of the log of fluorescence intensity was recorded as the regression coefficient.

When DAF values were plotted, the regression line was straight. On the other hand, when fluorescein sodium values were plotted, some regression lines were biphasic, representing an initial faster turnover rate and a subsequent slower turnover rate. In cases in which the turnover rate of fluorescein sodium became biphasic, the subsequent slower turnover rate was used as the flow rate.

All results are presented as the mean (SD). Statistical significance was calculated by comparing results using the Mann–Whitney test. A p value of <0.05 was considered statistically significant.

RESULTS

Calibration of DAF and fluorescein sodium

The relationship between fluorescence intensity and DAF concentration was linear (fig 2, $r^2 = 0.991$). The data generated by this method were consistent and reproducible. Fluorescence intensity was unaffected by the presence of 10% FBS (data not shown). Similar results were obtained when the fluorescein sodium standards were tested (data not shown).

Turnover rate

A representative result of turnover rate obtained from one subject is shown in fig 3. Fluorescence intensity of fluorescein sodium decayed with time at a flow rate of 14.5%/min. In the presented case, the fluorescence decay rate of DAF was much lower (1.14%/min) that that of fluorescein sodium. To test the reproducibility of the DAF method, the measurement was repeated three times in three eyes of three subjects. The coefficient of variance of the measurements was lower than 0.1 in all cases (mean = 0.07, n = 3), which ensured the reproducibility of the measurement.

The turnover rates of DAF and fluorescein sodium were 0.93 (0.36)%/min and 10.3 (3.7)%/min, respectively (table 1). The turnover rate of DAF was significantly lower than that of fluorescein sodium ($p<0.05$).

The turnover rate of DAF positively correlated with that of fluorescein sodium (fig 4; $r^2 = 0.87$, $p<0.05$).

DISCUSSION

We used fluorescein sodium and DAF to assess independently the turnover rates of aqueous and lipid layers of the tear film. To the best of our knowledge, this is the first study to address the turnover rate of the tear-film lipid layer.

There are potential limitations when interpreting the results of the present study, of which one is that DAF is a free-fatty-acid conjugate of fluorescein. Tear lipids are known to consist of various classes of lipids, such as wax esters, cholesterol, cholesterol esters, phospholipids, glycolipids and free fatty acids.[2 3 24] Because different classes of lipids have different biophysical properties, including molecular weight, hydrophobicity, viscosity and binding capacity to tear lipocalin,[24 25] the turnover of all lipids in tears may not be the same as that of DAF. Another limitation is the usage of a 5% DAF emulsion containing Tween 80. Although the addition of a surfactant was essential to make the emulsion of DAF, we considered it undesirable because it might disrupt the tear-film stability. We minimised the amount of Tween 80 in the emulsion, and the applied volume of DAF emulsion into the eye, to avoid the effect of a surfactant as much as possible.

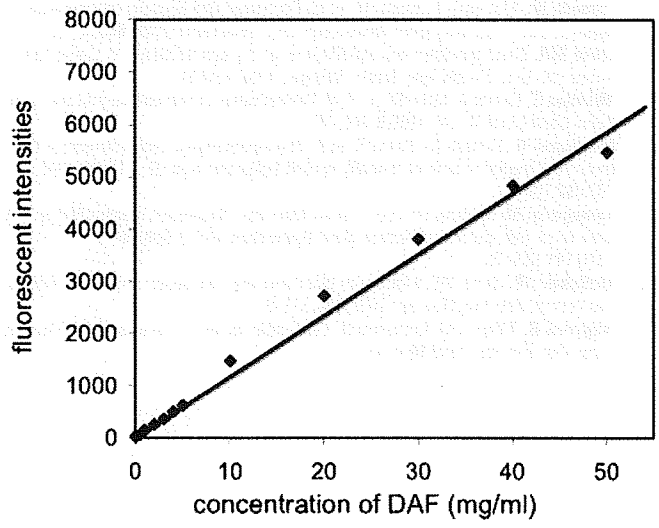

Figure 2 Plot of fluorescence intensity and 5-dodecanoylaminofluorescein (DAF) concentration. The relationship between fluorescence intensity and DAF concentration was linear ($r^2 = 0.991$).

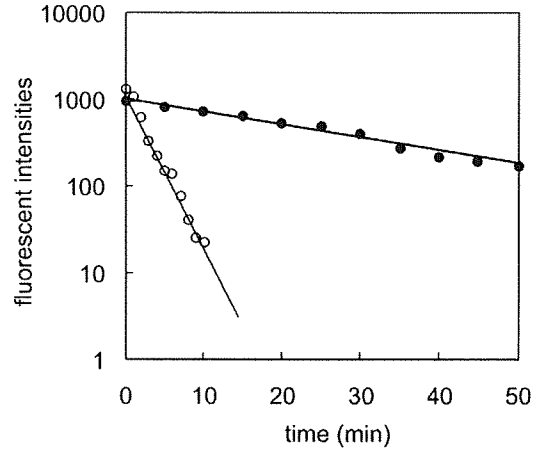

Figure 3 Representative result of turnover rate obtained from one subject. Fluorescence intensity of 5-dodecanoylaminofluorescein (black circle) decayed with time at a flow rate of 1.14%/min, which was lower than that of fluorescein sodium (14.5%/min; white circle).

The most interesting finding was that the turnover rate of DAF (0.9 (0.4)%/min) was approximately 9% that of fluorescein sodium (10.3 (3.8)%/min), indicating that the turnover of lipids in tears is much slower than the aqueous flow of tears. The discrepancy between the results of the dyes may be explained by the small bulk of the tear-film lipid layer compared with that of the marginal reservoirs.[2] Using meibometry, Chew *et al*[12 13] estimated that approximately 300 μg of lipid is present in the marginal reservoir and calculated that the preocular tear film contains approximately 9 μg of lipids. It has been estimated that the volume of the preocular tear film is 1–1.5 μl and that the total volume of tear fluid is 7–10 μl.[20] Our results confirm

Table 1 Turnover rates of a topically applied (1 μl) 50 mg/ml 5-dodecanoylaminofluorescein (DAF) emulsion or 50 μg/ml fluorescein sodium solution observed in 12 subjects

	Turnover rate (%/min)	
Subject no	DAF	Fluorescein sodium
1	0.51	5.2
2	0.46	4.4
3	0.54	7.1
4	0.61	7.2
5	0.84	9.7
6	0.93	10.0
7	0.84	10.2
8	1.12	11.5
9	1.60	14.7
10	1.31	12.9
11	1.24	15.8
12	1.14	14.5
Mean (SD)	0.93 (0.36)	10.3 (3.7)

The turnover rate of DAF was significantly lower than that of fluorescein sodium ($p<0.05$).