Serum- and Glucocorticoid-Regulated Kinase Isoform-1 and Epithelial Sodium Channel Subunits in Human Ocular Ciliary Epithelium

Saaeba Rauz,1,2 Elizabeth A. Walker,2 Susan V. Hughes,2 Miguel Coca-Prados,3 Martin Hewison,2 Philip I. Murray,1 and Paul M. Stewart2

PURPOSE. In peripheral sodium-transporting tissues, the serum- and glucocorticoid-regulated kinase (SGK) isoform-1 is an early corticosteroid target gene in the activation of epithelial sodium channels (ENaCs). Sodium transport across the human ocular nonpigmented and pigmented ciliary epithelial bilayer (NPE-PE) is essential for aqueous humor production, but the expression of SGK1 and ENaC subunits remain to be defined.

METHODS. SGK1 and ENaC subunits were evaluated by in situ hybridization and RT-PCR analysis on human NPE-PE sections and an NPE cell line (ODM-2). Northern blot analyses were conducted on ODM-2 cells incubated with dexamethasone (DEX) or aldosterone (ALDO) and RU38486 (a glucocorticoid receptor [GR] antagonist) or RU26752 (a mineralocorticoid receptor [MR] antagonist) or both inhibitors. The affinity of the GRs and MRs for DEX and ALDO was assessed by radioligand-binding assays.

RESULTS. Expression of SGK1 and ENaC subunits was confirmed in NPE-PE tissues and ODM-2 cells. Dose-dependent induction of SGK1 mRNA in the ODM-2 cells was demonstrated after incubation with DEX or ALDO. While response to DEX was not inhibited by RU38486 or RU26752, there was a moderate reduction in induction by ALDO in the presence of RU26752 that was completely abolished in the presence of both inhibitors. Specific binding of 3HDEX and 3HALDO was established, revealing greater expression of GRs than MRs.

CONCLUSIONS. The expression of ENaCs within the NPE-PE and corticosteroid regulation of SGK1 through the GR and MR, indicate that this mechanism may be a feature of sodium transport in the human ocular ciliary epithelium. (Invest Ophthalmol Vis Sci. 2003;44:1643–1651) DOI:10.1167/iovs.02-0514

One of the principal sodium-transporting tissues in the human eye is the ciliary epithelium. This is a complex bilayer of pigmented (PE) and nonpigmented (NPE) polarized, neuroepithelial cells oriented with apical surfaces opposed, allowing cell-to-cell communication through abundant gap junctions. Whereas the inner NPE layer lies in direct contact with the aqueous humor and is continuous with the neurosensory retina, the outer PE layer lies adjacent to the highly vascularized connective tissue stroma and is continuous with the retinal pigment epithelium. The primary function of the ciliary epithelium is the formation of aqueous humor, fundamental to the maintenance of intraocular pressure (IOP), and the provision of nutrition to the avascular and transparent structures of the eye, such as the trabecular meshwork, cornea, and lens.1 The translocation of ions and water across the ciliary epithelium, from the ciliary body stroma to the aqueous humor, is largely mediated by the energy-dependent Na\(^+\)K\(^+\) adenosine triphosphatase (ATPase) pump. Sodium and chloride ions are taken up from the stroma into the PE by a Na\(^+\)K\(^+\)2Cl\(^-\) cotransporter, and Cl\(^-\)/HCO\(_3\)\(^-\) and Na\(^+\)/H\(^+\) exchange.2–3 After diffusion into the NPE through the gap junctions, the ions are released into the aqueous humor through the Na\(^+\)K\(^+\)2ATPase pump,4–6 Cl\(^-\) and K\(^+\) channels,1,7 or an Na\(^+\)K\(^+\)2Cl\(^-\) cotransporter.8 The catalysis of OH\(^-\) to H\(^+\) and HCO\(_3\)\(^-\) by carbonic anhydrase is also crucial.9,10 Other mechanisms involved in aqueous humor formation include diffusion and ultrafiltration.1 A concomitant passive movement of water into the posterior chamber accompanies this sodium flux, and aquaporins appear not to be involved.10 Aqueous humor thus secreted into the posterior chamber, circulates between the iris and the lens, through the pupil, into the anterior chamber, where it is drained predominantly through the trabecular meshwork and uveoscleral outflow routes.

This mechanism is analogous to other tissues, such as the kidney and colon, where corticosteroids are known to play a key role in ion and water transport. In these target tissues, the isozyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), protects the mineralocorticoid receptor (MR) from cortisol by inactivation to cortisone,11 thereby allowing aldosterone (ALDO) to bind with high affinity to the MR. The active ligand-bound receptor complex translocates to the nucleus where it may dimerize with other ligand-receptor complexes, binding to hormone-response elements, inducing activation of target genes, and thereby initiating transcription and finally synthesis or repression of proteins that are ultimately responsible for the physiological effects of ALDO. One of these target genes is serum and glucocorticoid-regulated kinase (SGK) isoform-1,12–15 which induces activation of the epithelial sodium channel (ENaC; a heterotetramer consisting of 2\(\alpha\), 1\(\beta\), and 1\(\gamma\)-subunits) and the Na\(^+\)K\(^+\)2ATPase pump. The sodium transport response to mineralocorticoids is biphasic; an early phase commencing after a latent period of 30 to 45 minutes, with an increased apical membrane permeability mediated through the ENaC, and a late phase of several hours to days, possibly involving de novo synthesis of ENaC and basolateral Na\(^+\)K\(^+\)2ATPase.14–18 SGK1 has been identified as an early corticosteroid target gene that activates preexisting ENaCs.13,17,19,20 Three isoforms of SGK have

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been recognized,21,22 all inducing ENaC-mediated apical sodium transport, but only isoform 1 is sensitive to corticosteroids.

Recent studies have demonstrated the presence of the MR, glucocorticoid receptor (GR), and, somewhat surprisingly, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), a cortisol-generating isozyme, within the ocular ciliary epithelium.23–26 Systemic inhibition of 11β-HSD1 by carbenoxolone results in a reduction of IOP, providing further evidence that the human eye is a corticosteroid target tissue.26 Furthermore, preliminary data have confirmed expression of the α-ENaC subunit in the ciliary epithelium, and this subunit may be involved in cell volume regulation.27–29 In this study we defined expression of SGK1 and ENaC subunits in the human ocular ciliary epithelium and analyzed the corticosteroid regulation of SGK1 at this site using dexamethasone (DEX; a synthetic GR agonist), ALDO (an MR agonist), RU38486 (a GR antagonist), forskolin (a cAMP mimetic GR agonist), and genistein for the use of human tissue in research. Eyes were acquired at necropsy from DEPC-H2O, mounted on poly-L-lysine-coated slides (BDH, Poole, UK) according to the manufacturer’s protocol.

Reverse transcription of RNA was performed using a commercial system (Promega, Southampton, UK). A total of 1 μg RNA was preannealed with 0.75 μg random hexamers by incubation at 70°C for 5 minutes. Primer extension was performed at 37°C for 60 minutes after the addition of reaction buffer, 1 mM of each dNTP, 80 U rRNasin RNase inhibitor and 50 U avian myeloblastosis virus (AMV) reverse transcriptase. A 5-μL aliquot of this reaction was taken for subsequent PCR reactions using primer pairs for human SGK1 (Table 1). Amplification of a transcript size of 699 bp was performed with an initial denaturing step of 95°C, followed by 35 cycles of 95°C (1 minute), 60°C (1 minute) 72°C (1 minute), and a final elongation step of 72°C for 5 minutes. The reaction product was purified with a DNA purification system (Wizard PCR Prep; Promega), and the SGK1 sequence confirmed with an automated DNA sequencer (Applied Biosystems, Foster City, CA).

Human cDNA constructs for the α-, β-, and γ-subunits of the ENaC were generated by transforming full-length sequences of human α-ENaC, β-ENaC (both previously ligated to the pcMT3 vector), and γENaC (ligated to the pcDNA3 vector) subunits,32,33 into sub-cloning-efficiency DH5α-competent cells and amplified, and the plasmid DNA purified with a mini plasmid preparation kit (Totam Biologicals, Northampton, UK). Sequencing provided confirmation of the DNA insert. PCR was performed using a 1:10 dilution of the purified plasmid DNA with 3' and 5' primers designed to cover at least one intron–exon boundary for each human ENaC subunit (Table 1). Amplification of the relevant transcript size was performed using an initial denaturing step of 95°C, followed by 35 cycles of 95°C (1 minute), 54°C (1 minute) 72°C (1 minute), and a final elongation step of 72°C for 5 minutes. A 5-μL aliquot of each of the SGK1 and ENaC subunit amplified cDNA fragments, were ligated overnight at 4°C to a commercial vector (pGEM-T Easy; Promega), by using the 2X ligation buffer protocol. After transformation and amplification in sub-cloning-efficiency DH5α-competent cells, plasmid DNA was purified with a mini plasmid preparation kit (Totam Biologicals). Confirmation of the authenticity of the cDNA inserts was provided by DNA sequence analysis.

**Materials and Methods**

**Cell Culture**

ODM-2 cells (a human NPE cell line),30 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 1000 mg/mL stabilized glutamine (Glutamax; Gibco-Invitrogen Corp., Paisley, UK), supplemented with 10% (vol/vol) fetal calf serum (FCS), and were grown to 70% to 85% confluence at 37°C in 5% CO₂.

Human cortical collecting duct (HCD)31 cells were grown to 90% to confluence in DMEM/F12 with L-glutamine and HEPES (Gibco-BRL) supplemented with 2% (vol/vol) FCS and insulin-transferrin-selenium.

**Tissue Preparation**

Paraffin-embedded human ocular sections were obtained from the Academic Unit of Ophthalmology of the University of Birmingham and were managed according to the provisions of the Declaration of Helsinki for the use of human tissue in research. Eyes were acquired at surgical enucleation, and, in all cases, the underlying diagnosis was choroidal malignant melanoma. All sections were stained with hematoxylin and eosin and examined to ensure only adjacent normal anterior segment structures were studied. Using RNase-free conditions, the human cDNA constructs for the α-ENaC, β-ENaC, and γENaC subunits,32,33 into sub-cloning-efficiency DH5α-competent cells and amplified, and the plasmid DNA purified with a mini plasmid preparation kit (Totam Biologicals, Northampton, UK). Sequencing provided confirmation of the DNA insert. PCR was performed using a 1:10 dilution of the purified plasmid DNA with 3’ and 5’ primers designed to cover at least one intron–exon boundary for each human ENaC subunit (Table 1). Amplification of the relevant transcript size was performed using an initial denaturing step of 95°C, followed by 35 cycles of 95°C (1 minute), 54°C (1 minute) 72°C (1 minute), and a final elongation step of 72°C for 5 minutes. A 5-μL aliquot of each of the SGK1 and ENaC subunit amplified cDNA fragments, were ligated overnight at 4°C to a commercial vector (pGEM-T Easy; Promega), by using the 2X ligation buffer protocol. After transformation and amplification in sub-cloning-efficiency DH5α-competent cells, plasmid DNA was purified with a mini plasmid preparation kit (Totam Biologicals). Confirmation of the authenticity of the cDNA inserts was provided by DNA sequence analysis.

**Generation of SGK1 and ENaC cRNA Probes**

ENaC and SGK1 constructs were linearized using appropriate restriction enzymes generating 5’ overhangs and purified using the DNA purification system (Wizard PCR Prep; Promega). Antisense and sense complementary RNA probes were synthesized with a digoxigenin (DIG) labeling kit for SP6/T7 polymerase (Roche Molecular Biochemicals, Lewes, UK) and quantified using the DIG-luminescence detection kit (Roche Molecular Biochemicals).

**Generation of SGK1 and ENaC Plasmid DNA Constructs**

Human SGK1 cDNA constructs were generated by extracting RNA from a confluent 75-cm² tissue culture flask of HCD cells with a single-step extraction method (RNAlater B RNA isolation kit; AMS Biotechnology, Oxon, UK) according to the manufacturer’s protocol.

**Table 1. Primer Sequences Used for Generating cRNA and cDNA Probe Constructs**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>cDNA (bp)</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGK1</td>
<td>5’- AGGGCAGTTTTGGAAAGGTG 3’</td>
<td>699</td>
<td>XM_004255</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>5’- CAGCTCCAGTGTCTCTGCT 3’</td>
<td>601</td>
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<td>β-ENaC</td>
<td>5’- TTGCACACCAAGGAGCTATG 3’</td>
<td>1000</td>
<td>X87159.1</td>
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<tr>
<td>γENaC</td>
<td>5’- GTGCAAATCAGGAATCTTACA 3’</td>
<td>696</td>
<td>NM_001039.1</td>
</tr>
</tbody>
</table>
cDNA and nuclease-free water, respectively. RT-PCR, and the positive and negative controls were provided by HCD. All thermocycles commenced with an initial denaturation cycle at 95 °C for 5 minutes, followed by denaturation, annealing, and extension cycles, terminating with one final extension cycle at 72 °C for 5 minutes. Integrity of the RNA was confirmed by 18S ribosomal RNA RT-PCR, and the positive and negative controls were provided by HCD cDNA and nuclease-free water, respectively.

RT-PCR Analysis of SGK1 and ENaC Subunits in ODM-2 NPE Cells

RNA was extracted from a confluent 75-cm² tissue culture flask of ODM-2 cells, using a single-step extraction method (RNAzol B RNA isolation kit; AMS Biotechnology) according to the manufacturer’s protocol. After conducting the reverse-transcriptase reaction just described (generation of SGK1 and ENaC plasmid DNA constructs), a 5-μl aliquot was obtained for subsequent PCR reactions with the primer pairs for SGK1 and ENaC subunits (α, β, and γ) shown in Table 1. Optimal RT-PCR conditions required a 0.5-μg aliquot of cDNA template, and 0.5 mM MgCl₂ for all primer pairs (except the β-ENaC primer, 2.4 mM MgCl₂), and annealing temperatures of 60°C and 54°C for the SGK1 and α-, β-, and γ-ENaC oligonucleotide primer pairs, generating transcript sizes of 699, 601, 1000, and 696 bp, respectively. All thermocycles commenced with an initial denaturation cycle at 95°C for 5 minutes, followed by denaturation, annealing, and extension cycles, terminating with one final extension cycle at 72°C for 5 minutes. Integrity of the RNA was confirmed by 18S ribosomal RNA RT-PCR, and the positive and negative controls were provided by HCD cDNA and nuclease-free water, respectively.

In Situ Hybridization Analysis of SGK1 and ENaC Subunits in Human Ocular Ciliary Epithelium

Using RNase-free conditions, in situ hybridization (ISH) was performed on 5-μm paraffin-embedded sections of the anterior segment of six human eyes. Sections were preheated for 4 hours at 60°C, dewaxed, and permeabilized with 20 μg/ml RNase free proteinase K in 50 mM Tris-HCl, at 37°C for 20 minutes. After a rinse in 1× phosphate-buffered saline (PBS), sections were refixed at 4°C with 4% paraformaldehyde in PBS. Hybridization with antisense DIG-labeled cRNA probes (20–80 ng/100 μl) was performed at 20°C lower than the melting temperature for each probe for 16 hours in hybridization buffer (2.5× SSC, containing 62.5% deionized formamide [vol/vol] and 12.5% dextran sulfate [wt/vol]) and 120 μg/ml salmon sperm DNA. The sections were rinsed in DEPC-treated water, washed for 10 minutes at 25°C in 2× SSC, for 20 minutes at 50°C in 0.1× SSC, 60 minutes at 50°C in 0.05× SSC and 50% (vol/vol) deionized formamide, and 15 minutes at 25°C in Tris-buffered saline (TBS) with 1% bovine serum albumin (BSA). Hybridized DIG-labeled probes were detected after incubation at 37°C for 1 hour with anti-DIG alkaline phosphatase Fab fragments (750 U/ml) diluted 1:100 in 50 mM Tris-HCl. After final washes at room temperature in TBS-1% BSA, probes were visualized using 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) chromogen precipitation. To examine the PE in more detail, indirect fluorescence-ISH was performed by incubating the sections at 37°C overnight with 1:6 anti-DIG-fluorescein Fab fragments (Roche Molecular Biochemicals) prepared according to the manufacturer’s protocol. After final washes at 25°C in PBS-0.5% BSA, the sections were mounted in medium containing 4’6-diamidino-2-phenylindole (DAPI; Vectashield; Vector Laboratories, Peterborough, UK), and visualized with 494-nm (fluorescein) and 360-nm (DAPI) wavelength excitation filters, emitting 523-nm (yellow-green) and 460-nm (blue) fluorescence, respectively. In control experiments, antisense DIG-labeled cRNA probes in a 60-fold excess of unlabeled antisense cRNA probe, sense cRNA probes, or no probe was used.

Figure 1. In situ hybridization analysis of SGK1 and the ENaC subunits (α, β, and γ) in the nonpigmented ciliary epithelium (NPE). Incubation with antisense DIG-labeled cRNA probe demonstrated NBT/BCIP chromogen precipitation (blue-purple) representing SGK1 and ENaC subunit mRNA in the NPE (A–D), with minimal precipitation observed in the control sections: DIG-labeled antisense cRNA probe (E–H), and DIG-labeled sense cRNA probe (I–L). Magnification, ×400.

Figure 2. Indirect fluorescence-ISH of the NPE-PE bilayer. Expression of SGK1 (A) and ENaC subunits-α (B), -β (C), and -γ (D) were confirmed in the NPE. Fluorescence was masked in the central PE by pigment granules, but marked fluorescence was observed in the peripheral cytoplasm adjacent to the ciliary body. Incubation with SGK1 (E) and ENaC subunits-α (F), -β (G), and -γ (H) DIG-labeled sense cRNA probes revealed minimal fluorescence. Magnification, ×630.
Corticosteroid Regulation of SGK1 in ODM-2 Cells

Twenty-four hours before experimentation, ODM-2 cells were washed twice with 1× PBS to remove all traces of corticosteroid-containing serum, and the medium was replaced with MEM without phenol red, with 1000 mg/mL stabilized glutamine (Glutamax; Gibco-Invitrogen Corp.), supplemented with 10% (vol/vol) charcoal-stripped FCS (First Link, (UK) Ltd.) and 2 mM l-glutamine (Gibco-Invitrogen Corp.).

ODM-2 cells were treated with 10⁻⁷ M DEX (a synthetic steroid with almost exclusive affinity for GR) or ALDO (high affinity for the MR), diluted in serum-free MEM without phenol red, from a 10⁻⁴ M stock steroid solution in 100% ethanol, and incubated at 37°C in 5% CO₂. Total RNA was extracted at 0, 30, 60, 120, and 240 minutes after treatment, using the mammalian RNA extraction kit (Gen-Elute; Sigma, Poole, UK) according to the manufacturer’s protocol, but eluting the final RNA with 20 μL of elution buffer. RNA was stored at −70°C until further analysis.

Time-course experiments were performed to investigate the dose-response of SGK1 induction, with 10⁻⁶, 10⁻⁷, 10⁻⁸, and 10⁻⁹ M DEX or ALDO. To evaluate whether responses were mediated through either the GR or MR, ODM-2 cells were further treated with either 10⁻⁷ M DEX or ALDO in the presence of 100-fold excess of a GR antagonist (RU38486, mefipristone; Roussel Uclaf, Roumainville, France), an MR antagonist (RU26752; Roussel Uclaf), or both inhibitors.

Control experiments were performed in a similar manner but with the use of vehicle (100% ethanol) instead of the 10⁻⁷ M stock steroid solution, and subsequently diluted with serum-free MEM. All experiments were repeated at least five times.

Northern Blot Analysis

Aliquots of 10 μg denatured RNA were loaded per lane of a denaturing 1.5% formaldehyde-agarose gel and resolved by electrophoresis at 125 V for 3 to 4 hours, before transfer onto nylon filters (Hybond N+; Roche Molecular Biochemicals) overnight. After fixation by 254 nm UV irradiation, filters were incubated for 5 hours in prehybridization buffer containing 0.77 M sodium phosphate (pH7.2; 0.2 M NaH₂PO₄·H₂O + 0.58 M NaHPO₄) and 0.5 mM EDTA, 7% SDS (wt/vol), and 100 μg/mL denatured salmon sperm DNA (Sigma) and hybridized (18 hours) at 65°C with 3²P-]abeled SGK1 cDNA. Filters were washed to a final stringency of 0.5X SSC and 0.1% SDS at 55°C before autoradiography at −70°C for 2 days to 1 week. Filters were then stripped by adding a boiling solution of 0.1% SCC and 0.1% SDS directly onto the membrane, allowing the membrane to cool to room temperature on an orbital shaker, and repeating. This was followed by prehybridization and hybridization with 3²P-labeled RNA cDNA for 12 hours and washing to a final stringency of 0.1% SCC and 0.1% SDS at 65°C, before autoradiography at room temperature for 1 to 2 hours.

Densitometry was performed by capturing an image of the autoradiograph on computer (Gene Genius Bio-imaging System and Genesnap 4; Syngene-Synoptics, Ltd., Cambridge, UK) computer software. Quantification of the signal was also performed on computer (Genetools 3; Syngene-Synoptics Ltd.) and the data exported to a spreadsheet program (Excel 2000; Microsoft Corp., Redmond, WA) and a statistical analysis program (Minitab 13.1 Windows; Minitab Inc., State College, PA) for further analysis: expression of SGK1 mRNA was normalized to expression of 18S mRNA, and standardized to the base subunit–B for GR) or ALDO (high affinity for the MR), diluted in serum-free MEM without phenol red, from a 10⁻⁴ M stock steroid solution in 100% ethanol, and incubated at 37°C in 5% CO₂. Total RNA was extracted at 0, 30, 60, 120, and 240 minutes after treatment, using the mammalian RNA extraction kit (Gen-Elute; Sigma, Poole, UK) according to the manufacturer’s protocol, but eluting the final RNA with 20 μL of elution buffer. RNA was stored at −70°C until further analysis.

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**Radioligand-Binding Assays**

The capacity of glucocorticoid and mineralocorticoid binding was assessed by radiolabeled steroid binding assays, as previously described.³⁴ ODM-2 cells were grown to 80% to 90% confluence in 75-cm² tissue culture flasks, trypsinized, washed twice with 1× PBS, and resuspended in serum-free medium to achieve 5 × 10⁶ cells/mL. Aliquots (200 μL) of the cell suspension (1 × 10⁶ cells) were added to glass tubes containing increasing concentrations (0.1–20 nM) of (1) [²H]DEX (specific activity 89 Ci/mmol, Amersham Pharmacia Biotech UK, Ltd.); (2) [²H]DEX and a 200-fold excess of unlabeled DEX; (3) [²H]ALDO (specific activity 56 Ci/mmol, Amersham Pharmacia Biotech UK, Ltd.); (4) [²H]ALDO and a 200-fold excess of unlabeled ALDO; or (5) [²H]DEX and a 200-fold excess of RU38486.

Cells were incubated with the radiolabeled steroids for 1 hour at 37°C in 5% CO₂, washed twice with 500 μL of 1× PBS at 4°C, and centrifuged at 1500 rpm at 4°C for 10 minutes. This was followed by a final wash with 500 μL of 4°C lysis buffer (sucrose 0.25 M, Tris 0.02 M, Triton X-100 0.5% [vol/vol]; pH 7.4), and the final pellet was resuspended with 200 μL of 1× PBS at 4°C and 500 μL of absolute ethanol at 4°C. Bound radioactivity was analyzed by scintillation counting, and assays for Scatchard plots were performed in duplicate and repeated at least three times. Data were linearized by plotting specifically bound hormone divided by free hormone (total minus specifically bound hormone). The slope of the resultant Scatchard plot corresponded to the binding affinity value (dissociation constant, Kₒ), and the intercept with the y-axis corresponded to the total saturable binding value (maximal binding capacity, Bmax). By using the latter together with the Avogadro constant, it was possible to determine the number of GRs or MRs per cell.

**RESULTS**

ISH Analysis of SGK1 and ENaC Subunits in Human Ciliary Epithelium

ISH, with human SGK1 antisense cRNA probe and sections of the anterior segment of the human eye, showed expression of human SGK1 mRNA in the NPE when visualized by NBT/BCIP chromogen precipitation (Fig. 1A). There was some evidence of chromogen precipitation in the PE, and this was confirmed by indirect fluorescence-ISH (Fig. 2A). SGK1 mRNA expression was particularly intense in the peripheral cytoplasm adjacent to the ciliary body stroma, but fluorescence was masked by the pigment granules in the central PE cytoplasm. Control analyses with labeled SGK1 antisense cRNA with a 60-fold excess of unlabeled SGK1 antisense cRNA (Fig. 1E), DIG-labeled SGK1 sense cRNA (Fig. 1I), and no probe (data not shown), revealed minimal or no hybridization signal. Similarly, fluorescence-ISH...
control experiments with DIG-labeled SGK1 sense cRNA demonstrated minimal fluorescence (Fig. 2E).

All three ENaC subunit mRNAs were expressed in the NPE (Figs. 1B–D) and PE (Figs. 2B–D), and the pattern of distribution was similar to that of SGK1 mRNA. Control experiments revealed no or minimal hybridization signal (Figs. 1F–H, 1J–L; and Figs. 2F–H).

RT-PCR Analysis of SGK1 and ENaC Subunits in ODM-2 NPE Cells
SGK1 and \( \gamma \)-ENaC subunit mRNA species were consistently identified in the ODM-2 NPE cells (Fig. 3).

Corticosteroid Regulation of SGK1 mRNA Induction in ODM-2 Cells
Expression of SGK1 mRNA (a single band of 2.4 kbp) was rapidly induced in ODM-2 cells by \( 10^{-7} \) M DEX (twofold) at 30 minutes, was maximal (threefold) at 60 minutes, and declined by 240 minutes (Fig. 4A). When time course experiments were repeated with a range of DEX concentrations (\( 10^{-6} \)–\( 10^{-9} \)M), there was clear evidence of a dose response (Fig. 5), and at 240 minutes after incubation with \( 10^{-9} \) M DEX, expression of SGK1 mRNA had returned to baseline level. Experiments with a 100-fold excess of the GR and MR antagonists (RU38486 and RU26752, respectively), separately and together, failed to show inhibition of the SGK1 mRNA induction response to incubation with \( 10^{-7} \) M DEX, although the induction in the presence of both inhibitors was not statistically significant (Fig. 6).

SGK1 mRNA was induced to a similar extent by \( 10^{-7} \) M ALDO, but unlike \( 10^{-7} \) M DEX, expression returned to baseline level after 240 minutes of incubation (Fig. 4B). A dose response (ALDO \( 10^{-6} \)–\( 10^{-9} \) M) was also observed (Fig. 5), but in contrast to the SGK1 mRNA induction after \( 10^{-7} \) M DEX, RU26752 inhibited induction after \( 10^{-7} \) M ALDO, and complete inhibition was observed in the presence of both inhibitors (Fig. 6). No statistical significance was demonstrated when inhibitory effect of both inhibitors and RU26752 were compared at the 60-minute time point.

Control experiments performed by substituting the steroid for vehicle revealed no induction of SGK1 mRNA (Fig. 4C).

Analysis of GR and MR Expression in the ODM-2 Cells
ODM-2 NPE cells showed specific binding of both \([3H]DEX\) and \([3H]ALDO\) (Fig. 7A). Scatchard analysis of the binding kinetics showed that the \( B_{\text{max}} \) for \([3H]DEX\) (34,000 GRs per cell) was greater than that for \([3H]ALDO\) (4,200 MRs per cell). The mean \( K_{D} \)s were \( 8.1 \times 10^{-9} \) and \( 3.6 \times 10^{-9} \) M for the GRs and MRs, respectively. Saturation binding kinetics comparing \([3H]DEX\) binding in the presence of a 200-fold excess of unlabeled DEX or RU38486, confirmed the specificity of binding with both agents displacing \([3H]DEX\) from GR (Fig. 7B).

 Discussion

Several pieces of evidence indicate that mineralocorticoid mechanisms play a role in sodium transport across the NPE-PE

**FIGURE 4.** Corticosteroid induction of SGK1 mRNA in ODM-2 cells. Northern blot analyses revealed rapid stimulation of SGK1 mRNA by both \( 10^{-7} \) M DEX (A) and \( 10^{-7} \) M ALDO (B) to maximum levels at 60 minutes. Induction was not present in control (vehicle) experiments (C). Normalization of sample loading was assessed by subsequent reprobing of the nylon filters with \(^{32}P\)-labeled 18S cDNA probe. Results are expressed as multiples of change in SGK1 level. Data are expressed as the mean \pm SEM; \( n = 5; \*P < 0.05, \**P < 0.01, \***P < 0.001.\)**
bilayer. The MRs, GRs, and somewhat surprisingly, 11β-HSD1, together with the α-ENaC subunit, have been localized to the ocular ciliary epithelium.23–26 Studies in vitro suggest that the α-ENaC subunit may have a role in sodium reabsorption by the NPE,29 a phenomenon less widely investigated than sodium secretion into the posterior chamber. Studies in vivo performed on rabbits have demonstrated increased IOP after administration of ALDO and decreased IOP after spironolactone (an MR antagonist)35 and mifepristone (RU38486, a GR antagonist),36 whereas in human studies, IOP has been shown to decline after carbenoxolone (an inhibitor of 11β-HSD).26 Using ISH, we have successfully demonstrated expression of the α-ENaC subunit and have also defined expression of the β- and γ-ENaC subunits and SGK1 mRNAs to both the NPE, and by indirect fluorescence-ISH, the PE cytoplasm, although the full extent of expression at this latter site was masked by pigment granules. RT-PCR analysis confirmed expression of SGK1 and ENaC subunit in the ODM-2 NPE cultured cells. These data support the potential for corticosteroid regulatory mechanisms as possible contributors to the net secretion of sodium and aqueous humor formation.

The ENaC is a heterotetramer consisting of three subunits (α, β, and γ) in a ratio of 2:1:1. Expression of all subunits is needed for full activation of the channels, although expression of the α-ENaC subunit alone or in combination with the β- or γ-ENaC leads to generation of a small sodium flux.32,33 ENaC activity is induced by mineralocorticoids, and it is thought that the initial early sodium response is mediated through mechanisms such as direct phosphorylation of one or more of the ENaC subunits37 or interaction with neuronal precursor cells expressed developmental downregulated 4 (Nedd4)38 that increases ENaC stability at the cell surface. SGK1 plays a key role in ENaC function by increasing sodium flux and cell surface expression, but to date direct phosphorylation of the ENaC subunits by SGK1 has not been demonstrated. Nevertheless, a recent study has shown that SGK1 phosphorylates the Nedd4 intermediary protein, thereby regulating ENaC cell surface activity and expression.39 Subsequent ubiquitination of the ENaC-Nedd4 complex facilitates endocytosis and eventual lysosomal degradation.38 ENaC turnover is rapid, with a reported half-life of 40 to 120 minutes.38,40 As a result, ENaC subunits are almost undetectable by immunohistochemistry at the apical membrane in the absence of prior ALDO stimulation,18,41 whereas expression has been more successfully demonstrated by ISH both in cultured cells and tissues, or by fluorophore-labeled techniques.13,20,42

SGK1, a member of the serine-threonine protein kinase family, was first characterized as a glucocorticoid and serum regulated mRNA in a rat mammary epithelial tumor cell line.12 SGK1 mRNA levels are strongly and rapidly induced by a variety of regulators, including ALDO, DEX, follicle-stimulating hormone,43 vitamin D,44 osmotic stress,45 insulin, transforming growth factor-β,46 and SGK1-immunoreactive protein has been shown to be induced, peaking 6 hours after incubation with corticosteroids.15

**Figure 5.** Dose-dependent response of SGK1 mRNA in ODM-2 cells. ODM-2 cells were treated with 10⁻⁶, 10⁻⁷, 10⁻⁸, and 10⁻⁹ M DEX and ALDO. Densitometry of Northern blot analyses demonstrated dose-dependent SGK1 mRNA induction after incubation with both corticosteroids. Results are expressed as multiples of SGK1 induction ± SEM. Changes across the time course are shown; n = 5; *P < 0.05, **P < 0.01, ***P < 0.001.
We have confirmed induction of SGK1 mRNA by both DEX and ALDO in ODM-2 sodium-transporting human NPE cells, reaching a peak at 60 minutes. A dose-dependent induction was observed in response to ALDO that was reduced with the MR antagonist, RU26752. The response was completely abolished in the presence of both inhibitors. In contrast, induction by ALDO was reduced by $10^{-5}$ M RU38486 and $10^{-5}$ M RU26752, and completely abolished with both inhibitors. Results are expressed as multiples of change in SGK1 induction ± SEM. Changes across the time course are shown; $n = 5$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 6. Receptor specificity of corticosteroid induced SGK1 mRNA expression in ODM-2 cells. Densitometry of Northern blot analyses demonstrated percentage of inhibition of SGK1 mRNA induction by $10^{-7}$ M DEX in the presence of either $10^{-5}$ M RU38486 (GR antagonist), $10^{-5}$ M RU26752 (MR antagonist), or a 100-fold excess of both inhibitors. In contrast, induction by ALDO was reduced by $10^{-5}$ M RU38486 and $10^{-5}$ M RU26752, and completely abolished with both inhibitors. One explanation of our findings could be partly dependent on the rapid non-nuclear action of corticosteroid hormones that seems to be particularly important in the role of glucocorticoids in neural function (neurotransmitters, second-messenger systems, modulation of mood, and behavior) and also in glucocorticoid-mediated immune function. A two-step model for corticosteroid action has been developed in an attempt to explain early ($<10$ minutes) and late ($>10$ minutes) cellular responses to corticosteroids and consists of both rapid non-genomic and classic genomic modes of steroid action. In this model, it is proposed that the nongenomic pathway of steroid action involves membrane receptors, intracellular second messengers, and effector systems at the level of the plasma membrane. In addition, nuclear steroid-receptor complex-initiated nuclear transcription and protein synthesis are modulated by non genomic signaling cascades and ion transporter activities, and thus may be especially relevant in the physiological effects of mineralocorticoids. It remains to be seen whether the DEX-mediated induction of SGK1 mRNA in ODM-2 cells is.
dependent in part, on non-nuclear mechanisms or on yet uncharacterized mechanisms.

In summary, we have demonstrated expression and corticosteroid regulation of SGK1 mRNA and the ENaC subunits in the human ocular ciliary epithelium. The induction of SGK1 mRNA by ALDO appears to involve the MR, whereas induction by DEX appears to be mediated partly through GRs (or MRs), but possibly through additional nongenomic or uncharacterized routes. The corticosteroid regulation of SGK1 through GRs and MRs and expression of ENaC within the NPE-PE indicate that this mechanism may be an integral feature of sodium transport signaling cascade in the human ocular ciliary epithelium.

References

SGK1 and ENaC in Human Ocular Ciliary Epithelium


