Four Subunits ($\alpha\beta\gamma\delta$) of the Epithelial Sodium Channel (ENaC) Are Expressed in the Human Eye in Various Locations

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Purpose. The epithelial sodium channel (ENaC) is typically expressed in sodium-absorbing epithelia. Several reports suggest that ENaC is also expressed in ocular tissues and may play a role in aqueous humor secretion and glaucoma. However, the precise localization of ENaC in the human eye is still unclear. Here, the authors studied ENaC expression in 12 normal human donor eyes and in six eyes of patients with glaucoma.

Methods. Quantitative real-time PCR was used to investigate the expression of α-, β-, γ-, and δ-ENaC transcripts in ocular tissues. In addition, the authors performed immunohistochemical studies using recently generated antibodies against human β- and γ-ENaC.

Results. At the mRNA level, all four ENaC subunits were found to be expressed in a wide range of ocular tissues from normal and glaucomatous human eyes, with the cornea, ciliary body, iris, and retina showing the highest expression levels. At the protein level, β- and γ-ENaC subunits showed distinct distribution patterns and could be immunolocalized primarily to the cell membranes of epithelial cells of the cornea and to the conjunctiva, iris, ciliary body, lens, and retinal pigment epithelium but also to vascular endothelial cells, smooth muscle cells, stromal cells, and retinal neurons. The authors found no altered mRNA level of any subunit in glaucomatous eyes.

Conclusions. All four ENaC subunits ($\alpha\beta\gamma\delta$) are expressed in the normal human eye, with distinct localization of subunits possibly reflecting different functional states of the channel. The (patho-)physiological roles of ENaC in the various localizations in the eye remain to be determined. (*Invest Ophthalmol Vis Sci.* 2012;53:596–604) DOI:10.1167/iovs.11-8581

The epithelial sodium channel (ENaC) is a member of the ENaC/degenerin family of ion channels and is typically localized in the apical membrane of sodium-absorbing epithelial cells, such as in the distal nephron, distal colon, respiratory epithelia, and urinary bladder and in the ducts of sweat and salivary glands.[1–3] In these epithelia ENaC-mediated Na^+ influx across the apical membrane is the rate-limiting step for transepithelial Na^+ transport. Thus, appropriate regulation of ENaC plays an important role in the fine-tuning of transepithelial fluid and electrolyte transport and in the overall maintenance of the body's sodium balance. The hormonal signals, local mediators, and molecular mechanisms involved in ENaC regulation are highly complex and still incompletely understood (see Ref. 4 for review). Interestingly, ENaC expression has also been reported in skin, vascular endothelial and smooth muscle cells, and glial and neuronal cells, where its physiological roles remain to be determined.[5–12]

ENaC is a heteromultimeric channel usually composed of three homologous subunits (α, β, and γ) with a 30% to 40% identity at the level of their amino acid sequence.[13] Each subunit has a large extracellular domain, short intracellular amino- and carboxyl-termini, and two transmembrane domains (M1 and M2). The M2 domains are thought to contribute to the channel pore. Expression of α-ENaC alone or in combination with either β- or γ-ENaC is sufficient for channel activity to occur. In contrast, expression of β-ENaC or γ-ENaC alone does not result in measurable ENaC currents. Co-expression of all three subunits (α, β, γ) is needed for full channel activity.[13] A fourth subunit, δ-ENaC, has been cloned from a human kidney cDNA library with transcriptional expression in a range of tissues including testis, ovary, pancreas, and brain.[14,15] Genes corresponding to human δ-ENaC have been identified in chimpanzee, dog, chicken, and rabbit but seem to be absent in mouse and rat.[15,16] In heterologous expression systems, δ-ENaC has functional similarities to α-ENaC,[14,17] and it is more closely related to α-ENaC at the sequence level (37% amino acid identity) than it is to β- and γ-ENaC. Thus far, little is known about the physiological role of δ-ENaC. The available crystal structure of the related acid-sensing ion channel ASIC1[18] and recent atomic force microscopy studies[19] suggest that ENaC is a trimer. It is likely that both, the α-subunit and the δ-subunit, can co-assemble with the other two subunits to form functional $\alpha\beta\gamma$- or $\delta\beta\gamma$-ENaC.[17] However, the precise stoichiometry and subunit composition of ENaC remains a matter of debate and may vary in different locations.

In ocular tissues, a wide range of membrane transport mechanisms, including various types of ion channels, are involved in intracellular ion homeostasis and transepithelial ion transport. The latter determines the composition of intraocular fluids and provides the driving force for osmotically obliged fluid movement across ocular epithelia, which is important, for example, in the maintenance of the transparency of the cornea and lens, in aqueous humor formation, and in retinal function.[20–22] In this context it is of interest that evidence has been reported for ENaC expression in ocular tissues of different

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species, including rat, mouse, rabbit, ox, and human.[12,23–37] Ocular ENaC expression does not seem to be limited to epithelial cells but has been observed in fibroblasts,[33] retinal neurons, glial cells,[12,24] and photoreceptors.[28,29] In the human eye, ENaC expression has been detected in the cornea,[30,33,36] ciliary body,[26,34,35] and retina.[34] Little is known about the (patho-)physiological role of ENaC in the eye, but ENaC may contribute to aqueous humor formation,[26] corneal endothelial sodium and fluid transport,[31] and the pathophysiology of certain forms of glaucoma.[27]

A comprehensive study on ENaC expression, localization, and subunit distribution in the human eye is not yet available. Moreover, as far as we know, the expression of δ-ENaC has not yet been elucidated in the human eye. Therefore, in the present study, we investigated the transcriptional expression of α-, β-, γ-, and δ-ENaC subunits in various human ocular tissues using quantitative real-time PCR. Given that a previous study demonstrated specific transcriptional upregulation of α-ENaC in the retina in a mouse model of glaucoma,[27] we also studied the expression of ENaC transcripts in ocular tissues obtained from glaucoma patients. In addition, our recently established subunit-specific antibodies for human β-ENaC and γ-ENaC[17] allowed us to localize ocular ENaC expression by immunocytochemistry.

Materials and Methods

Tissues

Ocular tissues were obtained from 12 normal donor eyes without any known ocular disease and were used for real-time PCR (mean age, 79.0 ± 6.7 years; four women, two men) or immunohistochemistry (mean age, 67.5 ± 6.6 years; three women, three men). These eyes were obtained at autopsy and were processed within 10 hours of death. In addition, tissues of three eyes with open-angle glaucoma (mean age, 85.7 ± 3.7 years; two women, one man) and three eyes with angle-closure glaucoma (mean age, 80.0 ± 3.7 years; two women, one man) were used. These eyes had to be surgically enucleated because of painful absolute glaucoma and were processed immediately after enucleation. It is unusual that chronic open-angle glaucoma causes pain requiring enucleation of the affected eye. The three enucleated eyes with an anatomically open chamber angle used in this study were blind and painful eyes with a long-standing history of open-angle glaucoma and multiple surgical interventions, a cup to disc (C/D) ratio of 1.0, and intraocular pressure (IOP) levels up to 45 mm Hg. Clinical data on one of the open-angle glaucoma eyes used in the present study have previously been reported.[38] Informed consent to tissue donation was obtained from the patients or, in the case of autopsy eyes, from their relatives; the study was approved by the local Ethics Committee and adhered to the tenets of the Declaration of Helsinki for experiments involving human tissues and samples.

Antibodies

We recently generated and described subunit-specific rabbit polyclonal antibodies against human β- and γ-ENaC.[17] We confirmed that these antibodies recognize human β-ENaC and γ-ENaC heterologously expressed in HEK293 cells and in native human kidney tissue in a subunit-specific manner (Supplementary Data and Supplementary Figs. S1, S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8581/-/DCSupplemental).

Immunohistochemistry

Immunofluorescence labeling of ocular tissues was performed as previously described.[39] In negative control samples, the primary antibody was replaced by PBS or equimolar concentrations of nonimmune rabbit IgG. Specificity of the antibodies was also determined by preadsorption of the primary antibodies with the respective immunizing peptides (1 μg/mL) for 1 hour at room temperature before the staining procedure.

Quantitative Real-time RT-PCR

RNA extraction and cDNA synthesis was performed as previously described.[40] Quantitative real-time PCR was carried out with software and a thermal cycler (MyIQ Thermal Cycler; Bio-Rad, Munich, Germany). PCR reactions (25 μL) contained 2 μL cDNA, 3.5 mM $MgCl_2$, 0.2 μM each upstream and downstream primer, and 0.1 μM universal probe (Roche) in 1× supermix (iQ Supermix; Bio-Rad). All samples were analyzed in duplicate with a program of 95°C for 3 minutes and 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. For quantification, standard curves using serial dilutions (10^2-10^7 copies) of plasmid-cloned amplicons were run in parallel. For normalization of gene expression levels, ratios relative to the housekeeping gene *GAPDH* were calculated. Primer sequences (Eurofins, Anzing, Germany) are shown in Table 1.

Results

Expression of ENaC Transcripts in Human Ocular Tissues

Using quantitative real-time PCR, we demonstrated that at the mRNA level all four ENaC subunits (αβγδ) are expressed in the human eye, with α-ENaC consistently showing the highest expression levels (Fig. 1). Corneal tissue expressed high levels of α-ENaC and moderate levels of β- and γ-ENaC, whereas δ-ENaC was not expressed (Fig. 1A). Although much lower expression levels were observed in the trabecular meshwork, the relative expression pattern with the highest level of α-ENaC and lacking expression of δ-ENaC paralleled that of the cornea (Fig. 1B). A relatively balanced expression of α-, β-, γ-, and δ-subunits was found in the iris. However, the expression level of δ-ENaC was lower than for

Table 1. Primers Used for Quantitative Real-Time PCR

Gene	Accession No.	Product (bp)	Probe	Sequence (5′–3′)
GAPDH	NM_002046	66	60	AGCCACATCGCTCAGACAC
				GCCCAATACGACCAAATCC
α-ENaC	NM_001038	72	31	AACCAGGTCTCCTGCAACC
				GAAAGTATAGCAGTTTCCATAC
β-ENaC	NM_000336	60	15	GACCAAAGCACCAATATCACC
				GAAGTAGATGTTGAGCTTGAC
γ-ENaC	NM_001039	64	8	TCTACAACGCTGCCTACTCG
				TCCACCATCTTTGTCTGGAA
δ-ENaC	NM_002978	127	87	AGCCAGTGACGAAGCTGTG
				AAGCAGGATGGAGCCTCTG

The probe used was the TaqMan probe from the Universal Probe Library.

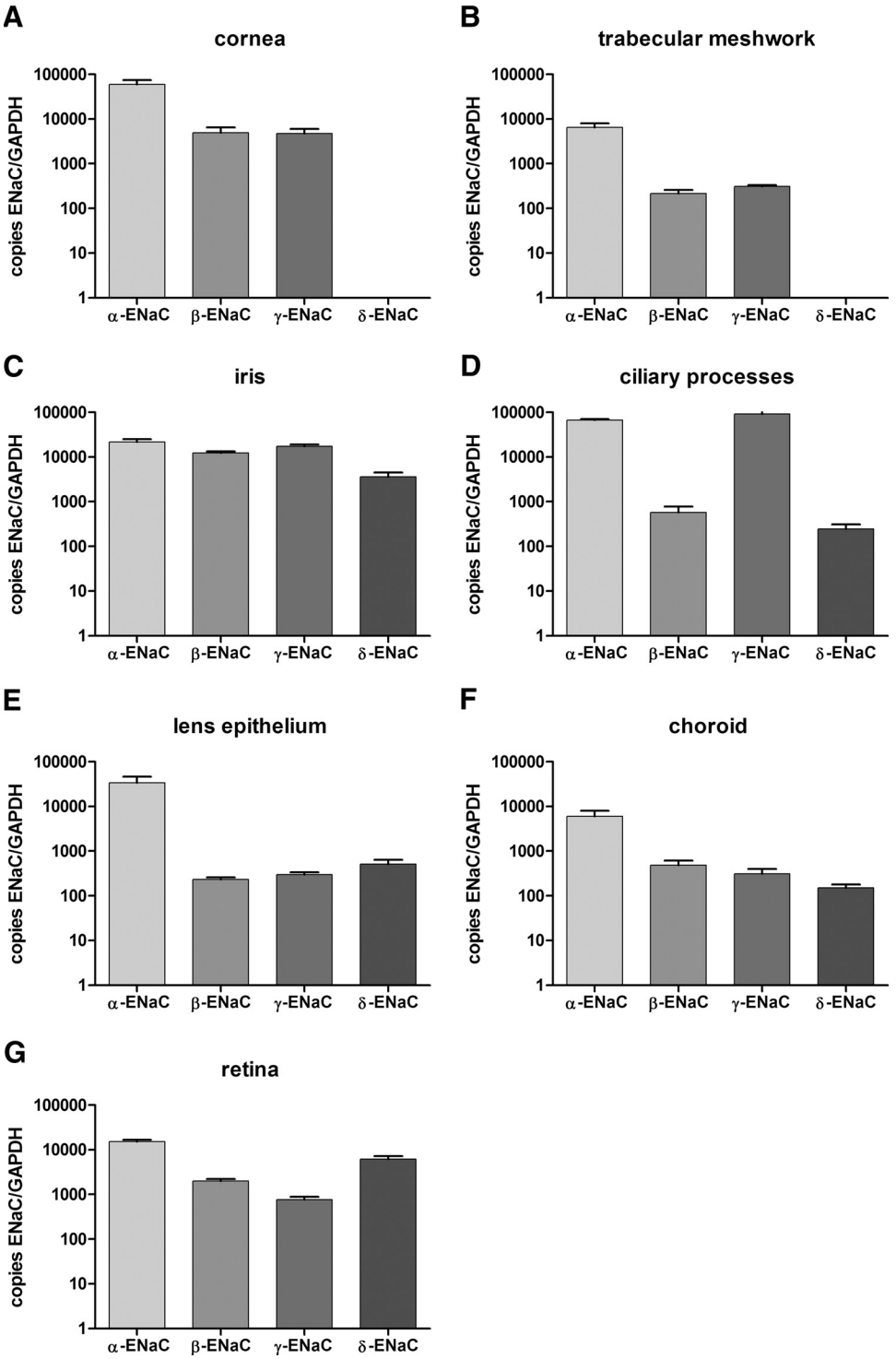

FIGURE 1. (**A–G**) Quantitative determination of mRNA expression levels of ENaC isoforms in different human ocular tissues using real-time PCR technology. The expression level was normalized against GAPDH, and the results are expressed as copy number ENaC per GAPDH. Values represent mean ± SD of three separate experiments.

the other three subunits (Fig. 1C). Ciliary processes revealed a pronounced expression of α- and γ-ENaC but only weak expression of β- and δ-ENaC (Fig. 1D). In the lens epithelium and choroid, prominent expression of α-ENaC and weak expression of all other subunits were observed (Figs. 1E, 1F). Finally, in the retina, expression of α-ENaC was strongest, with moderate expression of δ-ENaC and β-ENaC and weak expression of γ-ENaC (Fig. 1G).

The data shown in Figure 1 can also be used to compare the expression levels of each subunit in different ocular tissues. Expression of α-ENaC was strongest in ciliary processes and cornea, followed by the lens, iris, and retina, and weakest in the choroid and trabecular meshwork. In contrast, β-ENaC showed the highest expression levels in the iris, moderate levels in the cornea and retina, and weak levels in the ciliary processes, choroid, trabecular meshwork, and lens. The expression pattern of γ-ENaC was similar to that of β-ENaC, with the exception of the ciliary processes in which a very high expression level of the γ-subunit was observed exceeding even that of the α-subunit. The highest δ-ENaC expression was found in the retina, followed by the iris; weak expression was found in the lens,

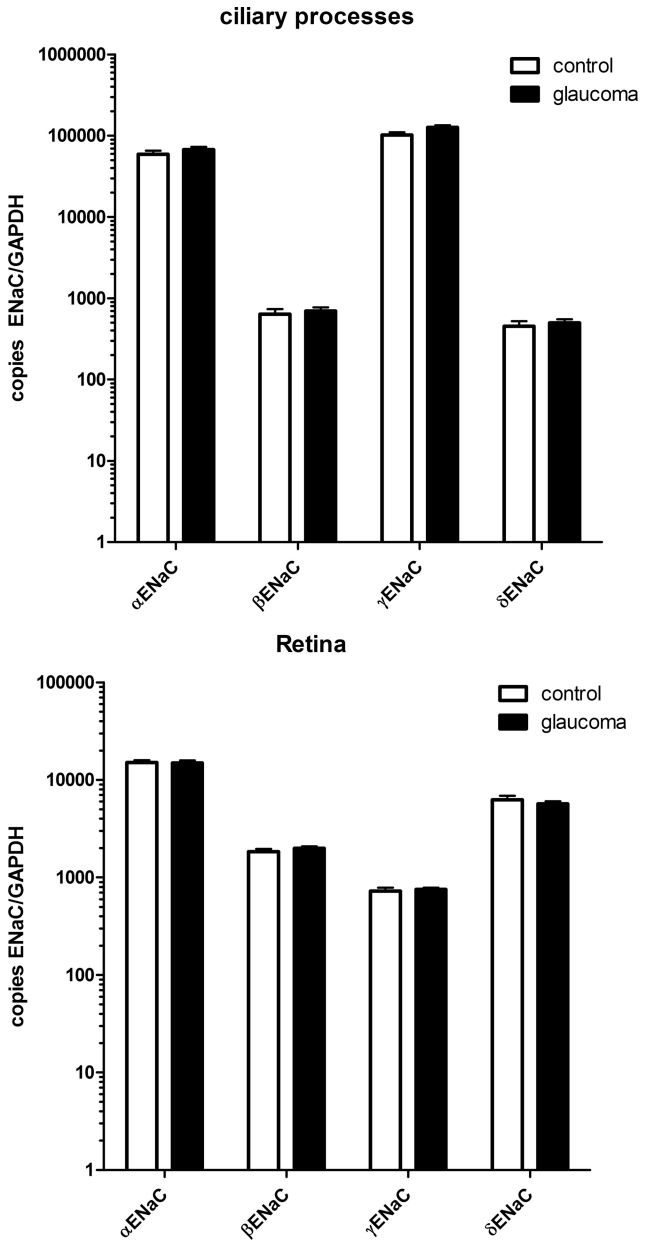

FIGURE 2. Quantitative determination of mRNA expression levels of ENaC isoforms in human ciliary processes (*top*) and retina (*bottom*) comparing normal donors (control) and glaucoma patients ($n = 6$ for each patient group) using real-time PCR technology. The expression level was normalized against GAPDH, and the results are expressed as copy number ENaC per GAPDH.

ciliary body, and choroid. Thus, subunit expression patterns varied considerably in different ocular tissues.

Comparison of ENaC Expression in Normal and Glaucomatous Eyes

In additional quantitative real-time PCR experiments, we investigated the expression of ENaC subunits in ocular tissues obtained from patients with glaucoma ($n = 6$) and compared that with the expression in normal control tissues ($n = 6$). No significant differences in expression levels of ENaC transcripts were observed in glaucomatous eyes compared with normal eyes (Fig. 2). Thus, in human glaucomatous eyes, we could not confirm a transcriptional upregulation of α-ENaC in the retina that has previously been reported in a mouse model of glaucoma.[27]

Localization of ENaC β- and γ-Subunits in Human Ocular Tissues

Using immunohistochemistry, we found ENaC β- and γ-subunits to be widely expressed in various ocular tissues of normal eyes ($n = 6$). Staining was particularly prominent along the plasma membranes of epithelial cells, but in some tissues it was also prominent in vascular endothelial, stromal, and neuronal cells. No fluorescence signal above background was detected when nonimmune serum or PBS was used instead of the primary antibody. In the ciliary processes, we showed exemplarily that preadsorption of antibodies with their respective immunizing peptides resulted in the abolishment of staining, confirming the specificity of immune reactions (see Figs. 5E, 5F).

Immunolocalization of ENaC in corneal and conjunctival tissue revealed a prominent but incongruent distribution of β- and γ-subunits in ocular surface epithelia: the β-subunit was detected only in the apical cells of the corneal epithelium (Fig. 3A), in basal cell clusters of the limbal epithelium (Fig. 3B), and in basal cells of the conjunctival epithelium (Fig. 3C). In contrast, the γ-subunit was found to be expressed throughout all layers of the corneal epithelium (Fig. 3D) and in the suprabasal epithelium at the limbus, sparing the β-ENaC–positive basal cell clusters (Fig. 3E). The γ-subunit was also expressed within ectopic islands of corneal epithelial cells dispersed within the negative conjunctival epithelium (Fig. 3F). Both subunits were immunolocalized to the corneal endothelium and the stromal keratocytes (Figs. 3A, 3D, insets).

Weak expression of both ENaC subunits in the trabecular meshwork was focally detected in endothelial cells of Schlemm's canal, whereas the trabecular endothelial cells appeared negative (Figs. 4A, 4B). In iris tissue, both the β- and γ-subunits were immunolocalized to the basal and apical aspects of the iris pigment epithelium and to nonepithelial cells such as smooth muscle cells of the dilator and sphincter muscles, endothelial cells of stromal vessels, and stromal cells, forming the anterior border layer (Figs. 4C, 4D). The lens epithelium showed a punctate staining pattern with antibodies against β-ENaC and intracellular staining for γ-ENaC (Figs. 4E, 4F).

In the ciliary body, β-ENaC was detected primarily in the ciliary epithelium, where it localized to the opposed apical membranes of both the nonpigmented epithelial (NPE) and the pigmented epithelial (PE) layers covering the ciliary processes (Figs. 5A, 5B). However, we cannot differentiate between a localization of ENaC in the apical membrane of PE cells versus its localization in the apical membrane of NPE cells. In fact, ENaC may be present in the apical membrane of both cell types. In addition, β-ENaC was observed along the basal cell membranes of the PE. γ-ENaC was present primarily along the basal membrane domains of the PE (Figs. 5C, 5D). Both subunits were also expressed in smooth muscle cells of the ciliary muscle (data not shown).

In the posterior segment of the eye, choroidal tissue revealed positive staining for β-ENaC in the vascular walls of larger blood vessels, particularly in smooth muscle and endothelial cells (Fig. 6A), and positive staining for γ-ENaC in endothelial cells of the choriocapillaries (Fig. 6B). The retinal pigment epithelium was positive only for the β- but not for the γ-subunit (Figs. 6A, 6B). In the neuroretina, the most prominent staining for β-ENaC was seen in individual neurons of the ganglion cell and inner nuclear layers and in the photoreceptor layer in the transition zone between inner and outer segments (Figs. 6C, 6E). The γ-subunit was expressed primarily in retinal cells, often revealing a nuclear or a perinuclear staining pattern, in retinal nerve fibers and in the inner and outer nuclear layers (Figs. 6D, 6F).

In preliminary immunocytochemical experiments we did not detect significant differences in the localization of β- and γ-ENaC expression in glaucomatous versus control eyes apart

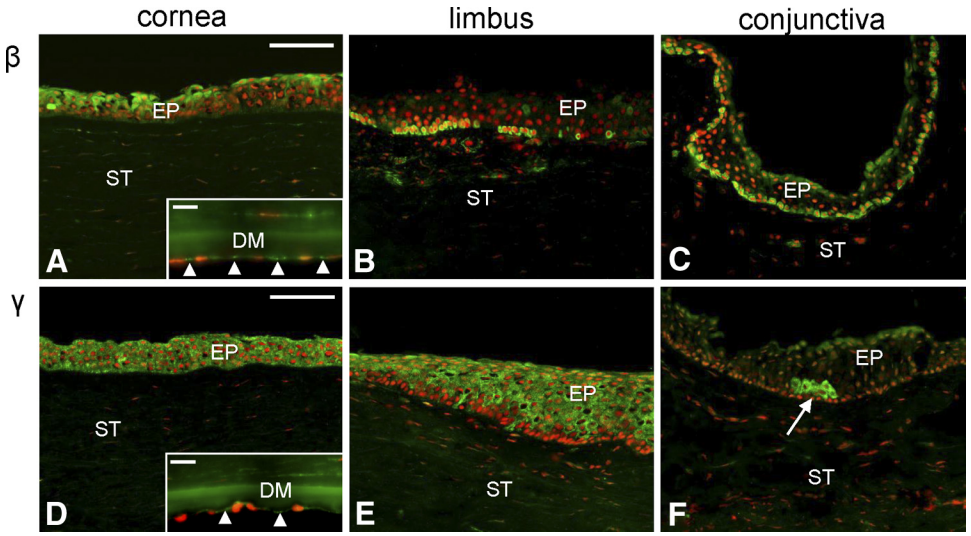

FIGURE 3. Immunolocalization of ENaC β- and γ-subunits in human cornea, limbus, and conjunctiva. The β-subunit is hardly detected in the corneal epithelium (**A**) but is present primarily in basal cells of the limbal (**B**) and conjunctival (**C**) epithelia and in corneal endothelial cells (*inset*, **A**, *arrowheads*). The γ-subunit is expressed in the corneal epithelium (**D**), the suprabasal epithelium at the limbus (**E**), and ectopic islands (*arrow*) of corneal epithelial cells in the conjunctival epithelium (**F**) and in the corneal endothelium (*inset*, **D**, *arrowheads*). DM, Descemet's membrane; EP, epithelium; ST, stroma. Scale bars: 100 μm (**A–F**); 20 μm (*insets*).

from an apparent lack of positively labeled retinal ganglion cells in the glaucomatous specimens (data not shown).

DISCUSSION

This study demonstrates the presence of all four ENaC subunits (αβγδ) in human ocular tissues, with a detailed analysis of the expression pattern of the different subunits. Using quantitative real-time PCR, we found ENaC expression in virtually all tissues of the anterior and posterior eye segments, with high expression levels in the cornea, ciliary body, iris, and retina and lower expression levels in the lens, trabecular meshwork, and choroid. Transcriptional ENaC expression was not different in glaucomatous eyes compared with normal eyes. In all ocular tissues expressing ENaC transcripts, we also obtained immunocytochemical evidence for ENaC expression by using recently established antibodies against human β-ENaC and γ-ENaC. Interestingly, our immunocytochemical data demonstrate ENaC expression not only in ocular epithelia but also in several nonepithelial cells, such as endothelial cells of blood vessels and in Schlemm's canal, smooth muscle cells of the iris and ciliary body, stromal cells of the iris anterior border layer, and retinal neurons.

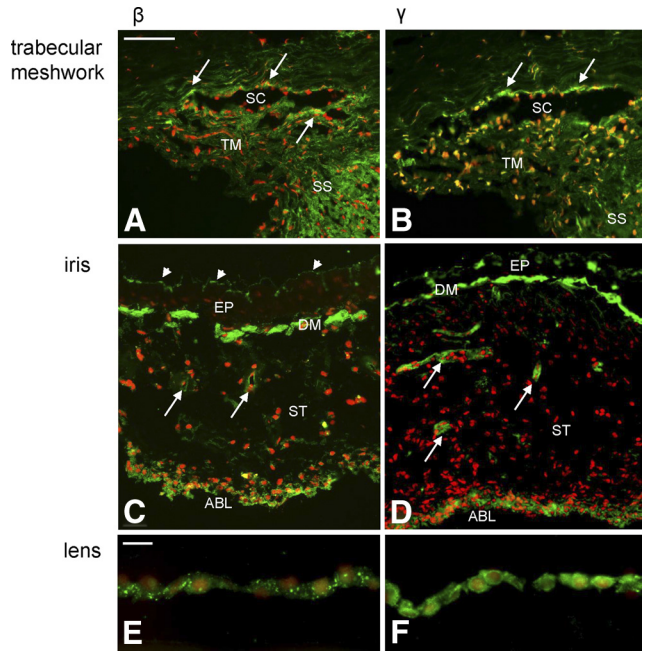

FIGURE 4. Immunolocalization of ENaC β- and γ-subunits in human trabecular meshwork (**A**, **B**), iris (**C**, **D**), and lens (**E**, **F**) tissue. (**A**, **B**) Both β- and γ-subunits can be focally detected in endothelial cells of Schlemm's canal (*arrows*) and in the scleral spur. (**C**, **D**) Both subunits can be immunolocalized to the basal aspects of the iris pigment epithelium (*arrowheads*), the dilator muscle, stromal vessels (*arrows*), and stromal cells forming the anterior border layer. (**E**, **F**) Both subunits are present in the lens epithelium showing a punctate staining pattern along cell membranes. ABL, anterior border layer; DM, dilator muscle; EP, epithelium; SC, Schlemm's canal; SS, scleral spur; ST, stroma; TM, trabecular meshwork. Scale bars: 100 μm (**A–D**); 20 μm (**E**, **F**).

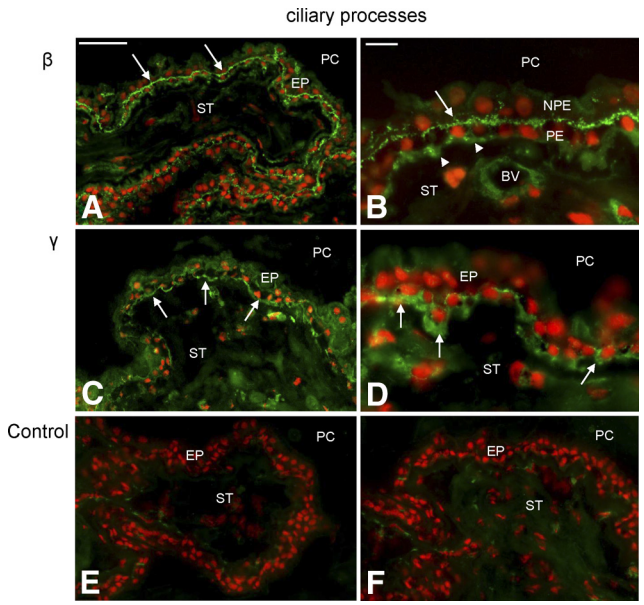

FIGURE 5. Immunolocalization of ENaC β- and γ-subunits in human ciliary processes. (**A**, **B**) The β-subunit can be immunolocalized to the opposed apical membranes of pigmented and NPE layers (*arrows*) and to the basal aspects of PE cells (*arrowheads*). (**C**, **D**) The γ-subunit is observed primarily along the basal aspects of the pigmented epithelial layer (*arrows*). (**E**, **F**) Preadsorption control experiments showing abolishment of specific staining for β- (**E**) and γ- (**F**) subunits. BV, blood vessel; EP, epithelium; PC, posterior chamber; ST, stroma. Scale bars: 100 μm (**A**, **C**, **E**, **F**); 20 μm (**B**, **D**).

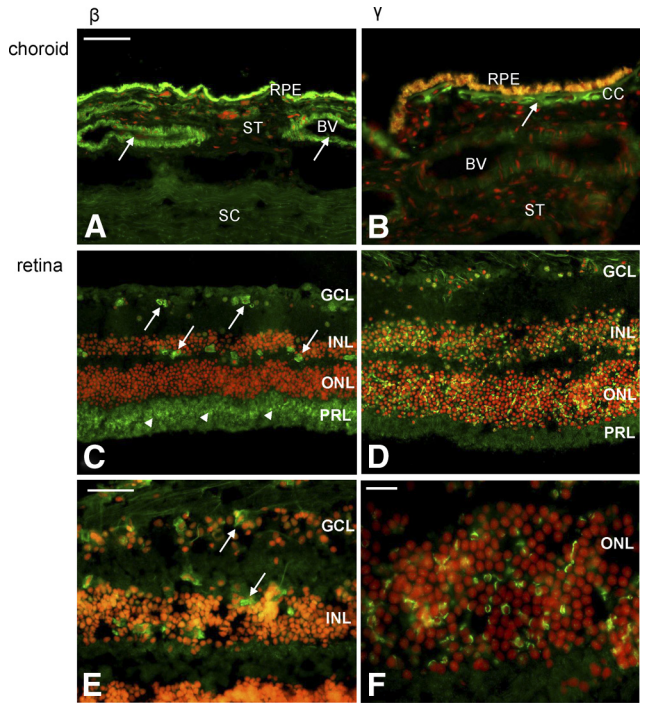

FIGURE 6. Immunolocalization of ENaC β- and γ-subunits in human choroid (**A**, **B**) and retina (**C**–**F**). (**A**, **B**) The β-subunit can be immunolocalized to the retinal pigment epithelium and to the walls of the large choroidal blood vessels (*arrows*, **A**), whereas the γ-subunit is expressed primarily in the choriocapillaries (*arrows*, **B**). (**C**–**F**) The β-subunit can be immunolocalized to individual neurons in the ganglion cell layer and inner nuclear layer (*arrows*) and in the photoreceptor layer (*arrowheads*, **C**, **E**). The γ-subunit is expressed in the ganglion cell layer, and in the inner and outer nuclear layers (**D**, **F**). BV, blood vessel; CC, choriocapillaris; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PRL, photoreceptor layer; RPE, retinal pigment epithelium; SC, sclera; ST, choroidal stroma. Scale bars: 200 μm (**A**–**D**); 100 μm (**E**); 20 μm (**F**).

Regional Subunit Distribution and Abundant Ocular Expression of α-ENaC Transcripts

We found α-, β-, and γ-ENaC mRNA, but not δ-ENaC mRNA, transcripts in the cornea and trabecular meshwork. This indicates that in these tissues, the αβγ configuration of ENaC is prevalent. In contrast, in iris, ciliary body, lens, and choroid, all four subunits (αβγδ) were detected. In all ocular tissues with ENaC expression, the α-subunit showed the highest mRNA levels, with the exception of the ciliary processes in which γ-ENaC mRNA was more abundant than α-ENaC mRNA. Interestingly, the α-subunit is thought to be essential for channel function,[13] and, in the absence of β- and γ-ENaC, homomeric channels consisting of α-subunits may be formed. Thus, the expression of α-ENaC alone is sufficient for relevant channel function that may be enhanced and modified by the coexpression of additional subunits.

Substantial Presence of δ-ENaC Transcripts in Human Eye

The presence of δ-ENaC in several human ocular tissues is a novel observation. In heterologous expression systems, the expression of δ-ENaC alone results in measurable ENaC currents that are enhanced by the coexpression of β- and γ-ENaC.[14] Thus, the δ-subunit is functionally similar to the α-subunit. Interestingly, in all ocular tissues expressing δ-ENaC, the mRNA level of α-ENaC was higher than that of the δ-subunit, which suggests a predominance of the α-subunit over the δ-subunit in the eye. The highest expression of the δ-subunit was detected in the retina. This suggests that in the retina, a substantial population of channels have a δβγ configuration rather than the classical αβγ configuration. Channels containing a δ-subunit instead of an α-subunit display some distinctive properties. For example, δβγ-ENaC is more than one order of magnitude less sensitive to amiloride than αβγ-ENaC.[14,17,41,42] Furthermore, δβγ-ENaC, but not αβγ-ENaC, can be activated by extracellular protons.[43,44] Another difference is the higher single-channel Na^+ conductance (~12 pS vs. ~5 pS)[14] and the reduced self-inhibition by extracellular Na^+[42] of δβγ-ENaC compared with αβγ-ENaC. Therefore, the presence of α- and δ-subunits indicates that ENaC exists in different functional states in different parts of the eye.

Incongruent Cellular Localization of β- and γ-ENaC

Interestingly, the immunocytochemical staining pattern of β-ENaC was different from that of γ-ENaC. At first sight this incongruent expression pattern may be surprising. However, in classical ENaC-expressing tissues such as kidney, colon,[45] and lung, it is well known that individual ENaC subunits are differentially regulated with tissue-specific expression patterns and with different responsiveness of individual subunits to hormonal regulation. Thus, the degree of constitutive and regulated expression may be different for each channel subunit and may vary from tissue to tissue and within tissues between cell types. From these observations the concept of "noncoordinate regulation" of ENaC subunits has evolved.[46,47] Heterologous coexpression of αβ and αγ subunits results in measurable channel activity with unique single-channel properties.[48,49] Therefore, it is conceivable that tissue-specific subunit expression patterns may be a regulatory mechanism to adjust the properties of ENaC to specific functional needs. Moreover, we must consider the possibility that ENaC subunits coassemble with other members of the ENaC/degenerin ion channel family (e.g., the acid-sensing ion channel ASIC1).[50] This may result in different channel properties and may add a level of complexity to ENaC regulation. Interestingly, ASIC expression has been reported in the eye and may be important for retinal function.[51]

Epithelial αβγ Configuration of ENaC in Corneal Endothelial and Epithelial Cells

The cornea is thought to maintain its hydration and thickness by a pump-leak mechanism, in which the endothelial monolayer maintains both a barrier to fluid movement from the anterior chamber into the stroma and an active pump of fluid out of the stroma into the aqueous humor.[52–54] This net fluid efflux from the stroma across the corneal endothelium depends on sodium and bicarbonate ion transport by way of several ion transporters and channels, allowing water to passively follow. Our finding that ENaC is present in corneal endothelium is in good agreement with previous reports.[25,31] Recirculation of a fraction of paracellular Na^+ flux may occur through apical ENaC and may be important for corneal endothelial fluid transport by electroosmosis.[31]

Active Na^+ absorption from the tear to the stromal side by the corneal epithelium has been demonstrated in several corneal preparations from different species.[20] The finding that Na^+ absorption across corneal epithelial cells can be blocked by ENaC-specific inhibitors suggests an involvement of ENaC.[55] This conclusion is further supported by molecular evidence of ENaC expression in corneal epithelial cells.[32–34,36,56] ENaC expression has also been reported in rabbit and human conjunctival tissue.[30] Interestingly, in the latter study, topical application of amiloride was shown to increase the quantity of

preocular tears, possibly as a result of reduced ion and fluid absorption caused by ENaC inhibition.

The present study shows α-, β-, and γ-, but not δ-, ENaC mRNA expression in the human cornea. Thus, ENaC is likely to exist in an αβγ conformation, which is typical for epithelial function and supports the concept that ENaC contributes to Na^+ transport in the corneal endothelium, corneal epithelium, and conjunctiva.

Prominent β-ENaC Expression in Putative Limbal Stem and Progenitor Cells

Using immunohistochemistry we confirmed the presence of β- and γ-ENaC in the corneal endothelium and found a noncongruent distribution pattern of both subunits in ocular surface epithelia. Interestingly, the β-subunit was present primarily in basal regions of the limbal epithelium, resembling stem and progenitor cell clusters.[57] Moreover, the β-subunit was found in basal cells of the conjunctival epithelium. Double-labeling experiments showed colocalization of β-ENaC, with putative stem and progenitor cell markers (ABCG2, p63α) in the basal cell clusters at the limbus (data not shown), suggesting a role of the β-subunit for limbal stem cell function. In contrast, immunostaining for the γ-subunit was observed throughout all layers of the corneal epithelium but not in the putative limbal stem cell population. This is an interesting observation and suggests that preferential expression of β-ENaC versus γ-ENaC may be linked to the state of cell differentiation. It is interesting that the modulation of ENaC activity may play a role in regeneration and wound healing.[5,25,58,59]

Potential Roles of ENaC in Aqueous Humor Formation and Outflow Regulation

The primary function of the double-layered ciliary epithelium, comprising pigmented and nonpigmented layers, is the secretion of aqueous humor,[22] which is essential for the maintenance of intraocular pressure and the provision of nutrients to avascular structures of the eye. The driving force for aqueous humor secretion is provided by transepithelial ion transport of Na^+, Cl^- and HCO_3^- generating an osmotic gradient for water movement.[22,60] Ion uptake by the PE cells is followed by diffusion of ions from PE to NPE cells through gap junctions, and ion release from the NPE cells into the posterior chamber.[61] In the present study, we immunolocalized β-ENaC primarily to the opposed apical membranes of PE and NPE layers of ciliary processes and, to a lesser extent, to the basal cell membranes of the PE facing the ciliary stroma. In contrast, γ-ENaC was present primarily along the basal membrane domains of the PE. As discussed, the functional relevance of this incongruent localization of both subunits is not yet clear. However, expression of the α-ENaC subunit in combination with either the β- or the γ-subunit is sufficient to generate a significant sodium flux.[48,62] Thus, strict cellular colocalization of the β- and γ-subunits is not required for relevant channel function provided that α-ENaC is present. In the ciliary epithelium this is likely to be the case, as suggested by our finding that α-ENaC transcripts are abundantly expressed in the ciliary body. Previous studies also have found ENaC expression in ciliary epithelium, consistent with our findings.[26,32,34,35] It has been speculated that ENaC may support reabsorption of Na^+ from the aqueous humor back into the NPE cells.[26] Alternatively, ENaC may facilitate Na^+ uptake from PE cells by the apical membrane of NPE cells which is then secreted by the Na^+/K^+-ATPase at the basolateral membrane facing the aqueous humor. From our immunocytochemical studies we cannot deduce whether ENaC is localized in the apical membrane of NPE or PE cells or both. ENaC expression in the apical membrane of PE cells would be difficult to reconcile with a role of ENaC in aqueous humor secretion. In contrast, the apparent expression of ENaC along the basal membrane domains of PE cells may indicate a role of ENaC in Na^+ uptake into PE cells. However, with our present data we are not yet in a position to propose a coherent model for the role of ENaC in aqueous humor secretion.

Interestingly, intraocular pressure is increased after prolonged exposure to mineralocorticoids and is reduced by the aldosterone antagonist spironolactone.[63,64] A significant decrease in intraocular pressure was also observed in glaucoma patients after treatment with spironolactone for 2 weeks.[65] In the kidney and colon, the mineralocorticoid aldosterone is the main hormonal stimulus of ENaC activity.[4] Therefore, stimulation of ENaC-mediated sodium transport across the ciliary epithelium by mineralocorticoids may contribute to the pathophysiology of glaucoma.[26] It should be mentioned that ENaC and related degenerin proteins have been reported to be mechanosensitive in glaucoma and may play a role in sensing endothelial shear stress, mechanical stiffness,[66] and blood pressure.[6] Therefore, ENaC may play a role in sensing intraocular pressure. Moreover, ENaC expression in endothelial cells lining Schlemm's canal may indicate a role in monitoring and modifying outflow resistance. However, at this stage no experimental data are available to support this hypothesis.

ENaC and Retinal Function

In good agreement with our findings, several previous studies have presented evidence of ENaC expression in the retina, with localization in the retinal pigment epithelium (RPE), photoreceptors, and neuronal cells of all retinal layers.[12,23,24,27–29,34,37] As mentioned, the human retina is the ocular tissue with the highest expression level of δ-ENaC transcripts. δ-ENaC has a broader tissue distribution than the other three subunits (αβγ) and may be present in neurons.[8] Therefore, retinal δ-ENaC may be localized with preference in retinal neurons and photoreceptors. Unfortunately, suitable antibodies that specifically recognize human δ-ENaC are not yet available to confirm this. In excitable cells, depolarizing Na^+ influx by ENaC may modify the resting membrane potential and, hence, the excitability of the cells.

In DBA/2J mice, a model for secondary angle-closure glaucoma that develop elevated intraocular pressure with subsequent loss of retinal ganglion cells, upregulation of α-ENaC has been observed in the neurosensory retina, both in synaptic and in nuclear layers.[27] At present it is unclear whether upregulation of ENaC is a general phenomenon in glaucoma or is limited to this mouse model. In our study, we could not detect any significant differences in expression levels of human ENaC transcripts in retinal or ciliary body tissues derived from patients with glaucoma compared with normal control specimens. However, we investigated only a limited number of end-stage glaucomatous eyes enucleated because of painful open-angle or angle-closure glaucoma. The pathophysiological situation in early glaucoma may be different, but human glaucoma eyes at an early disease stage were not available to us. At present we cannot rule out the possibility that differences in transcriptional ENaC expression may occur in earlier stages of open-angle or angle-closure glaucoma. Moreover, channel activity is not only determined by transcriptional expression but also by translation, post-translational modification, and protein trafficking. Thus, the findings presented in this study do not preclude ENaC activity to be altered in glaucoma.

Interestingly, the mineralocorticoid receptor has been detected in several ocular tissues.[12,29,33,63,67] ENaC has been detected in Müller glial cells[12,28,37] and RPE,[29] in which the mineralocorticoid receptor may colocalize with ENaC. Moreover, aldosterone has been shown to upregulate ENaC expression in Müller glial cells[12] and in the RPE.[29] A recent study further highlights the mineralocorticoid-sensitivity of rat neu-

roretina.[37] Whether the effects of aldosterone on retinal function are at least in part caused by mineralocorticoid receptor–mediated stimulation of ENaC remains to be determined.

Conclusion

In the human eye, the rather heterogeneous distribution pattern of the four ENaC subunits ($\alpha\beta\gamma\delta$) suggests that transmembrane sodium transport by ENaC may have a wide range of functional roles. These may include contributions to the maintenance of transparency and the hydration of cornea and lens, aqueous humor formation and regulation of intraocular pressure, and photoreception and neuronal processing in the retina. It is interesting that exposure to systemic or topical steroids is associated with a variety of ocular diseases and that ocular tissues express not only glucocorticoid receptors but also mineralocorticoid receptors. Because mineralocorticoids and glucocorticoids are the main hormonal regulators of ENaC in epithelial tissues, it is tempting to speculate that modifying ENaC function may contribute to the pathophysiology of steroid-induced ocular diseases. Thus, the use of ENaC inhibitors or blockers of the mineralocorticoid receptor may offer new therapeutic approaches for the treatment of ocular diseases. Therefore, future functional studies of the (patho-)physiological roles of ENaC in the eye are needed and are likely to be rewarding.

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