Control of Outflow Resistance by Soluble Adenylyl Cyclase

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Abstract

Glaucoma is a leading cause of blindness in the United States affecting as many as 2.2 million Americans. All current glaucoma treatment strategies aim to reduce intraocular pressure, even in patients with normal tension glaucoma. Typically, this is accomplished by reducing the rate of aqueous flow by limiting aqueous production or enhancing drainage using drugs and surgery. Whereas these strategies are effective in diminishing vision loss, some patients continue to lose vision and many discontinue use of their medications because of undesirable side effects. Drugs known to be effective in altering conventional outflow have for the most part been abandoned from modern clinical practice due to undesirable side effects. Identification of new drugs that could enhance conventional outflow, would offer additional options in the treatment of glaucoma and ocular hypertension. To this end, our laboratory has recently uncovered a novel pathway for regulation of conventional outflow by the ciliary body. This pathway is dependent on soluble adenylyl cyclase, an enzyme that catalyzes the generation of cyclic adenosine 3',5' monophosphate (cAMP) in response to bicarbonate.

Introduction

ACCORDING TO THE MODIFIED Goldmann equation, intraocular pressure (IOP) is a function of aqueous production (F_a), outflow through both conventional (C_t) and uveoscleral (F_u) pathways, and episcleral venous pressure (EVP) as follows:

$$\text{IOP} = \text{EVP} + (F_a - F_u)/C_t.$$

C_t occurs through the trabecular meshwork (TM) with the fluid eventually leaving the eye through the Schlemm's canal.¹ Elevated IOP accompanies approximately half of all glaucoma cases and lowering IOP is the only accepted treatment available to slow disease progression. This can be accomplished surgically, or as is more frequently the case, using topically applied pharmaceuticals. Drugs used to lower IOP typically reduce inflow by decreasing F_a or outflow resistance. Today, most drugs decrease the outflow resistance target F_u .^{2,3} However, the ratio of C_t to F_u varies with age, and F_u is not the main path for drainage of aqueous humor.^{4,5} Interestingly, some of the earliest drugs used to treat glaucoma (eg, pilocarpine) increase outflow through the conventional pathway.⁶ However, these drugs are not normally prescribed long term as they exhibit side effects. The development of drugs that could specifically increase C_t without significant side effects would substantially increase the pharmaceutical options for lowering IOP. In this study, we detail our serendipitous finding of a pathway for the

physiological regulation of C_t that may be amenable to development of such drugs.

Bestrophins

Bestrophins are a family of integral membrane proteins that function as anion channels and regulators of Ca^{2+} signaling.^{7,8} There are 4 bestrophins in the human genome, 2 of which are known to be expressed in the eye.^{9–11} Bestrophin-1 is exclusively expressed in the retinal pigment epithelium and select glial cells in the brain.^{12,13} Mutations in bestrophin-1 have been causally associated with 5 distinct hereditary retinal degenerative diseases (there are no known systemic neuronal complications).⁷ Bestrophin-2 (Best2) is expressed in a number of organs, including the colon, parotid gland, sweat gland, olfactory, and the eye.^{11,14–16} In the eye, Best2 is found exclusively in the nonpigmented epithelium (NPE) of the ciliary body (CB).^{11,17} We have shown that Best2 functions as a bicarbonate channel.¹⁵ This combined with its localization in NPE cells caused us to examine IOP in *Best2*^{-/-} mice. Although we worked under the hypothesis that absence of Best2 would cause increased IOP due to an increase in intracellular bicarbonate in NPE cells, we found the opposite; that *Best2*^{-/-} mice have a significantly lower IOP than their wild-type (WT) littermates.^{17,18} A more detailed examination of aqueous dynamics in the mice revealed that they do indeed produce more aqueous humor than their *Best2*^{+/+} littermates in a given span of time.¹⁸ However, C_t and F_u are increased sufficiently to overcompensate for the

increase in aqueous production. Since Best2 is found only in the CB,¹⁸ and there were no noticeable anatomic changes in the anterior chamber of the *Best2*^{-/-} mice, we concluded that there must be a mechanism for pressure-independent, biochemical communication between the inflow and outflow pathways. To understand how this pathway might work, we considered that Best2 functions as a bicarbonate channel. In 1993, Mittag et al.¹⁹ reported a bicarbonate-sensitive adenylyl cyclase (AC) activity in the rabbit CB. Reasoning that a bicarbonate-sensitive enzyme in the NPE could function downstream of Best2, we focused on this bicarbonate-sensitive AC activity.

Soluble Adenylyl Cyclase: A Bicarbonate Sensor

ACs are enzymes that catalyzes the conversion of adenosine-5'-triphosphate (ATP) to cyclic adenosine 3',5' monophosphate (cAMP). In mammals, there are 10 distinct AC genes. Nine of these encode transmembrane adenylyl cyclases (tmACs). The tmACs have differential expression patterns, are stimulated by forskolin, and physically and functionally interact with G-protein-coupled receptors (GPCRs).²⁰⁻²²

Of the AC genes, *ADCY10* encodes the most evolutionarily conserved, but most recently identified member of the AC family; soluble adenylyl cyclase (sAC).²⁰⁻²² Due to its shorter history and a lack of blockbuster medical drugs targeting sAC pathways, sAC is not as well known as the tmACs. Soluble AC activity was first reported in 1975.²³ In the testis, a soluble source of AC activity was identified and predicted to be attributable to an enzyme unique from the well-known tmACs (Fig. 1). However, the enzyme was not isolated nor the gene cloned until 1999.²¹ There are at least 2 sAC isoforms generated through alternative splicing that have different regulative characteristics.²⁴ sAC is expressed ubiquitously throughout different cell types. sAC can be localized anywhere in the cell, even within an organelle.^{22,25,26} sAC, unlike tmACs, is not sensitive to G-proteins and forskolin.²¹ It is involved in sperm capacitation and in other cell functions as a bicarbonate sensor generating cAMP in response to bicarbonate.²⁷ The ability of sAC to function as a bicarbonate sensor suggested that sAC may function downstream of Best2 in the NPE.

sAC Expression in the CB and TM

Following up on the work of Mittag et al.,¹⁹ we performed cAMP forming assays and found that the CB of both human and pig contains a high level of bicarbonate-sensitive AC activity. The sensitivity of this activity to the sAC-specific inhibitor KH7 strongly suggested that the enzyme responsible is sAC.²⁸ RT-PCR amplification using primer sets corresponding to 2 different regions of sAC cDNA clearly demonstrated sAC mRNA expression in the mouse CB (Fig. 2). Using sAC-specific antibodies, we found that the sAC protein is expressed in human and pig CB. Immunofluorescent localization of sAC in the eye demonstrated that it is highly expressed in the mouse NPE and stroma of the CB and in other ocular tissues such as the corneal endothelium.^{28,29} As shown in Figure 2, however, we did not observe the expression of sAC in drainage tissues of the mouse, specifically the TM and Shlemm's canal by immunofluorescence.²⁸

FIG. 1. Two sources of cyclic adenosine 3',5' monophosphate (cAMP) in the mammalian cells. Membrane-based adenylyl cyclases (transmembrane adenylyl cyclase [tmAC]) are activated by G proteins and forskolin. Soluble adenylyl cyclase (sAC) is not a membrane protein, is found in a variety of intracellular locations, and is activated by bicarbonate but not forskolin.

Similarly, we could not detect sAC in TM by western blot or immunoprecipitation, nor could we detect any KH7-inhibitable AC activity in pig TM lysates.²⁸

Interestingly, sAC was highly expressed in the CB, specifically in NPE where Best2 is also localized to the basolateral plasma membrane.²⁸ The finding that a bicarbonate-sensitive AC was highly expressed in the same cells as the bicarbonate channel Best2, but not in drainage tissues, caused us to further examine the effect of sAC on outflow facility to determine whether it is a mediator of communication between the CB and drainage tissues.

Physiological Effects of sAC on Outflow Resistance

To test the hypothesis that sAC in the CB is a regulator of C_t , we administered the sAC-specific inhibitor KH7 to mice and measured changes in IOP and EVP. We found that mice receiving KH7 showed a >40% increase in IOP, but no increase in EVP. *Best2*^{-/-} mice injected with KH7 exhibited a >45% increase in IOP-EVP in comparison to controls.²⁸ Again, no change in EVP was observed. These data led us to conclude that the effect of KH7 was specific to aqueous flow.²⁸ To determine why IOP was increased, we assessed the effect of KH7 on inflow and outflow in mice. We found that KH7 caused ~50% decrease in C_t compared with controls.²⁸ However, there was no statistically significant change in inflow. F_u was increased ~16% in KH7-treated mice in comparison to controls, although this difference was considered to be within the range of our experimental error.²⁸ These results indicated that sAC in the CB plays a significant role in regulation of C_t . However, there was still a possibility

FIG. 2. sAC is expressed in the ciliary body (CB), but not drainage tissues. **(A)** Mouse CB contains mRNA for sAC as evidenced by RT-PCR **(A)** using primer sets designed to span multiple exons covering 2 different regions of sAC. Bands matched those obtained from testis (T) and were absent in water controls (neg). Immunofluorescence staining for sAC **(B)** demonstrates that it is highly expressed in the CB, where it colocalizes with the nonpigmented epithelium (NPE)-specific protein Bestrophin-2 (Best2) **(C)**. **(D)** Detailed examination of a merged fluorescence and differential interference contrast image demonstrates that sAC is highly expressed in the CB, where it is concentrated in the NPE cells and stroma, but is not observed in pigment epithelium cells. Whereas sAC is also expressed in corneal endothelia, epithelia, and retina, no specific sAC staining was noted in drainage tissues. (This figure was originally published in *The Journal of Biological Chemistry*. See reference 28 © the American Society for Biochemistry and Molecular Biology.) Color images available online at www.liebertpub.com/jop

FIG. 3. Potential mechanism of outflow resistance control by sAC in the CB. Outflow resistance could be regulated by controlling the level of cAMP in the AH. cAMP in the aqueous humor could affect outflow by conversion to adenosine by phosphodiesterases, interaction with an as yet unknown cAMP receptor, or uptake using an anion transporter such as the organic anion transporter 1 (OAT-1) triggering structural changes that could accommodate better drainage of AH through the trabecular meshwork (TM) and Schlemm's canal (SC).

that these observations resulted from nonspecific effects of KH7. Therefore, we turned to sAC knockout mice.

We studied *Sacy*^{tm1Lex/tm1Lex} mice, which were genetically engineered, to remove the C1 catalytic domain of sAC.²⁴ *Sacy*^{tm1Lex/tm1Lex} mice exhibited a higher IOP and lower outflow facility than WT controls, and KH7 had no significant effect on these parameters in *Sacy*^{tm1Lex/tm1Lex} mice.²⁸ These results clearly indicate that sAC is a critical regulator of IOP and provide very strong evidence for the existence of a biochemical pathway for communication between the CB and drainage tissues that is regulated by HCO₃⁻ and cAMP. The effects of sAC inhibition on IOP and C_t represent the clearest evidence to date that a communicative pathway must exist between the CB and drainage tissues for the specific purpose of regulating C_t independent of pressure.

A Model for Regulation of Outflow Facility by the CB and Future Directions

The idea that a pathway for regulation of outflow that originates in the CB is not new.³⁰ However, data supporting the existence of such a pathway were previously limited to the finding that CB cells express and presumably secrete neuropeptides into the aqueous humor.³⁰ Our initial finding that *Best2*^{-/-} mice have an increased *F_a*, but a decreased IOP due to enhanced drainage, was the first physical evidence that a pathway for regulating IOP originating in the CB exists.¹⁸ Our data demonstrate that bicarbonate stimulates the sAC activity in the NPE. Bicarbonate is thought to drive the production of aqueous humor by fueling Cl⁻ uptake in the pigment epithelia through Cl⁻/HCO₃⁻ exchange. When bicarbonate levels are high in the NPE cells, the aqueous flow is high. Under these conditions, we predict that sAC is maximally stimulated, causing the NPE to secrete factors into the aqueous humor that signal an increase in C_t to accommodate the increase in the aqueous humor. Figure 3 depicts a possible mechanism for regulating outflow resistance by the CB. It is likely that any increase of bicarbonate due to high demand for cell respiration in the PE and NPE activates sAC resulting in an increase in cellular cAMP. Accumulated cAMP could work by further signaling within the NPE/PE cell inducing secretion of signals into the aqueous humor, or the cAMP could enter the aqueous humor through a channel as has been observed in other organs.³¹ It is known that there is a high level of cAMP in the aqueous humor although its origin is currently unknown.^{32–35} If cAMP in the aqueous humor is the signal, then it most likely is converted to adenosine by phosphodiesterases. A role for adenosine in regulation of outflow resistance has been well documented.^{36–38} Others although less likely possibilities include interactions with an as yet unknown cAMP receptor, or uptake using an anion transporter such as organic anion transporter 1.^{39,40} In the latter case, the cAMP would act as an intracellular signal in the TM decreasing outflow resistance and thus lowering IOP.

Although cAMP functions as an extracellular signal in *Dictyostellium*, where it triggers the formation of the fruiting body,⁴¹ there is no known direct role for cAMP as an extracellular signal in the animal kingdom. For this reason, we favor scenarios in which cAMP generated by sAC either signals secretion of a different factor from the NPE such as a neuropeptide that functions as the messenger between the CB and TM,³⁰ or that cAMP in the aqueous humor is further

metabolized to adenosine, a molecule known to regulate outflow facility through GPCRs present in TM cells.^{32,33} Further characterization of this pathway will increase our understanding of the regulation of outflow facility, and potentially provide new avenues for the development of novel glaucoma therapeutics.

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Author Disclosure Statement

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