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Regulation of Anterior Chamber Drainage by Bicarbonate-sensitive Soluble Adenylyl Cyclase in the Ciliary Body*

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Glaucoma is a leading cause of blindness affecting as many as 2.2 million Americans. All current glaucoma treatment strategies aim to reduce intraocular pressure (IOP). IOP results from the resistance to drainage of aqueous humor (AH) produced by the ciliary body in a process requiring bicarbonate. Once secreted into the anterior chamber, AH drains from the eye via two pathways: uveoscleral and pressure-dependent or conventional outflow (C_t). Modulation of "inflow" and "outflow" pathways is thought to occur via distinct, local mechanisms. Mice deficient in the bicarbonate channel bestrophin-2 (Best2), however, exhibit a lower IOP despite an increase in AH production. Best2 is expressed uniquely in nonpigmented ciliary epithelial (NPE) cells providing evidence for a bicarbonate-dependent communicative pathway linking inflow and outflow. Here, we show that bicarbonate-sensitive soluble adenylyl cyclase (sAC) is highly expressed in the ciliary body in NPE cells, but appears to be absent from drainage tissues. Pharmacologic inhibition of sAC in mice causes a significant increase in IOP due to a decrease in C_t with no effect on inflow. In mice deficient in sAC IOP is elevated, and C_t is decreased relative to wild-type mice. Pharmacologic inhibition of sAC did not alter IOP or C_t in sAC-deficient mice. Based on these data we propose that the ciliary body can regulate C_t and that sAC serves as a critical sensor of bicarbonate in the ciliary body regulating the secretion of substances into the AH that govern outflow facility independent of pressure.

Glaucoma is a leading cause of blindness in the United States affecting as many as 2.2 million Americans (1). All current glaucoma treatment strategies aim to reduce intraocular pressure (IOP),[3] even in patients with "normal tension" glaucoma (2). It has long been recognized that IOP is the quotient of the rate of aqueous humor (AH) production (inflow) divided by the drainage resistance (outflow). As such, all therapies applied to date have sought to either diminish inflow or increase outflow using drugs and surgery. Carbonic anhydrase inhibitors, drugs that inhibit enzymes catalyzing hydration of CO_2 to H^+ and HCO_3^-, have been used for many years to treat glaucoma (3, 4). However, the role of CO_2/HCO_3^- in regulating aqueous flow is poorly understood (5, 6). It is generally accepted that a transepithelial Cl^- flux drives the generation of AH with net transport occurring across the ciliary body (CB) epithelia in the stromal to aqueous direction (6). Uptake of Cl^- by the pigment epithelium is thought to occur at least in part by parallel Cl^-/HCO_3^- and Na^+/H^+ exchangers. The relative importance of these mechanisms varies from species to species, but the source of H^+ and HCO_3^- driving the exchangers is the CO_2 generated by respiration and its hydration by carbonic anhydrase in the pigment epithelium and the nonpigmented epithelium (NPE).

Bestrophins are a family of integral membrane proteins (7–9) that function as anion channels and regulators of Ca^{2+} signaling. Recently, we demonstrated that bestrophin 2 (Best2) functions as a HCO_3^- channel in colon goblet cells (10). In the eye, Best2 is uniquely localized to the basal membrane of NPE cells (11, 12). *Best2*$^{-/-}$ mice have a significantly lower IOP than their wild-type (WT) littermates (11, 13). Interestingly, this lower IOP occurs despite an increase in aqueous flow (F_a). The lower IOP results from overcompensation in conventional (C_t) and uveoscleral (C_u) drainage. The absence of Best2 in outflow tissues suggests the existence of pressure-independent, biochemical communication between the inflow and outflow pathways (13).

In trying to understand how absence of a HCO_3^- channel could affect inflow and outflow we considered that HCO_3^- would most likely be increased in the NPE of *Best2*$^{-/-}$ mice. This would result in an increase in aqueous flow as observed, but would not explain the effects observed on drainage in *Best2*$^{-/-}$ mice. Soluble adenylyl cyclase (sAC) serves as a HCO_3^- sensor in many tissues (14, 15). sAC catalyzes the formation of cAMP in response to increasing HCO_3^-. In 1993, Mittag *et al.* (16) reported a bicarbonate-sensitive adenylyl cyclase activity in rabbit CB. Here, we confirm that CB processes contain a high level of bicarbonate-sensitive adenylyl cyclase activity, and we show that sAC is expressed in the NPE and the stroma of the CB process. Administration of KH7, a specific inhibitor of sAC activity (17), caused a marked increase in IOP due to a decrease in C_t with no effect on C_u or F_a in WT

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[3] The abbreviations used are: IOP, intraocular pressure; AH, aqueous humor; Best2, bestrophin-2; CB, ciliary body; C_t, conventional outflow; C_u, uveoscleral outflow; EVP, episcleral venous pressure; F_a, aqueous flow; NPE, nonpigmented ciliary epithelium; sAC, soluble adenylyl cyclase; TM, trabecular meshwork; tmAC, transmembrane AC.

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mice. *Sacy*$^{tm1Lex/tm1Lex}$ mice, deficient in sAC, exhibited a higher IOP and lower C_t than WT mice, and KH7 had no effect on these parameters in *Sacy*$^{tm1Lex/tm1Lex}$ mice. These data demonstrate that sAC is a critical regulator of IOP and provide strong evidence for the existence of a biochemical pathway for communication between the CB processes and drainage tissues that is regulated by HCO_3^- and cAMP.

EXPERIMENTAL PROCEDURES

Assay for Bicarbonate-sensitive Adenylyl Cyclase Activity—Ciliary bodies were dissected from pig or human eyes and stored at −80 °C. Ciliary bodies were homogenized in 5 volumes of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM DTT) using a Dounce homogenizer on ice. Homogenates were centrifuged (10 min, 3000 × *g*, 4 °C), and the supernatant was assayed for cAMP forming activity. Aliquots were incubated in assay buffer (100 mM Tris, 2.5 mM ATP, 10 mM $MgCl_2$, 0.5 mM IBMX, 20 mM creatine phosphate, and 100 U^*ml^{-1} creatine phosphokinase) for 20 min (pig) or 50 min (human) at 30 °C, in the presence of 40 mM NaCl or $NaHCO_3$, with 50 μM KH7 or vehicle (dimethyl sulfoxide) (total volume 100 μl). Reactions were stopped by addition of an equal volume of 0.2 N HCl, and cAMP concentration was measured using an ELISA-based kit (AssayDesigns). Protein concentration in each sample was measured using a commercial kit (Bio-Rad), and cAMP produced was standardized protein content in each sample.

Immunoprecipitation—Identification of sAC in human and porcine tissue by immunoprecipitation was performed using a modification of the method of Farrell *et al.* (18). Human donor eyes were obtained from Donor Network of Arizona within 24 h postmortem. Following dissection, ciliary bodies (CB), trabecular meshwork (TM), brains, and corneas were stored at −80 °C. For immunoprecipitation, tissues were homogenized in 50 mM Tris, pH 8.0, 150 mM NaCl, 0.4 mM EDTA, 0.1 mM DTT, 1% Nonidet P-40, 1 mM PMSF, and 1 μl ml protease inhibitor mixture III (Calbiochem). Homogenates were subjected to a centrifugation at 45,000 × *g* for 30 min at 4 °C and supernatants collected. Protein concentration in each supernatant was determined using the BCA Protein assay (Bio-Rad), and equivalent amounts from each supernatant were diluted to 10 ml in homogenization buffer. Immunoprecipitation was performed using monoclonal antibody R37 as described elsewhere (18).

For Western blot analysis, immunoprecipitates were resuspended in 50 μl of SDS-PAGE sample buffer and resolved on 10% SDS-PAGE. Following transfer to PVDF membranes, sAC was identified by Western blotting with biotinylated monoclonal antibody R21 and detected using streptavidin conjugated to alkaline phosphatase.

Reverse Transcription PCR—RT-PCR was performed to confirm the expression of sAC mRNA in the mouse CB. Total RNA was isolated using TRIzol (Invitrogen) and reverse transcribed using Superscript III (Invitrogen) according to the manufacturer's protocols. The following primers sets were used: forward 5′-GCTTGTTTGAAGCCAAGGAG-3′ and reverse 5′-TTTCTCATTGAGGCCCAAAC-3′ for exons 5–11; forward 5′-CAGAAGCAACTGGAAGCCCTG-3′ and reverse 5′-CTTGTCCCGGATTTCCTGAGGCTG-3′ for exons 15 and 16; forward 5′-GGAGAAGATCTGGCACCACAC-3′ and reverse 5′-GGTGACCCGTCTCCGGAGTCC-3′ for β-actin. For positive control, testes tissue was used, and a reaction without cDNA template was used as negative control.

Immunofluorescent Staining—Mouse eyes were embedded in OCT (Tissue-Tek®) and 10-μm sections cut using a cryostat (Leica). Cryosections of the anterior segment of the eye were fixed with 4% paraformaldehyde in PBS for 10 min after which the tissue was denatured with 1% SDS as described previously (20). After blocking with PBS containing 5% BSA, sections were stained for sAC with biotinylated mAb R21 (1:500) and Best2 using polyclonal antibody B4947A (generously provided by Dr. H. Criss Hartzell, Emory University). sAC was visualized using a streptavidin-Alexa Fluor 568 conjugate, and Best2 was visualized using Alexa Fluor 488 goat anti-rabbit igG. Nuclei were labeled with 4,6′-diamidino-2-phenylindole (DAPI) (Sigma). Sections were examined and photographed using a Nikon E-600 microscope with CCD camera and ACTII software.

Measurement of AH Dynamics—Experiments to examine the effect of sAC inhibitor KH7 were performed on 3–6-month-old C57BL/6, *Best2*$^{-/-}$, or *Sacy*$^{tm1Lex/tm1Lex}$ mice back-crossed 10 generations onto a C57BL/6 background. sAC inhibitor KH7 was suspended in dimethyl sulfoxide and injected intraperitoneally in mice at a concentration of 0 or 5 μmol/kg. IOP and aqueous dynamics measurements were performed 3–4 h later and always between 2:00 and 6:00 p.m. to avoid diurnal pressure variations. Measurement of IOP, episcleral venous pressure (EVP), F_a, and C_t were made by cannulation of the anterior chamber with borosilicate glass microneedles as described previously (13, 21) in mice anesthetized with Avertin (300 mg/kg injected intraperitoneally). C_u was calculated using measured parameters according to the modified Goldmann equation: $C_u = F_a - C_t \times (IOP - EVP)$.

Statistical Analysis—Adenylyl cyclase activity is shown as mean ± S.E. and was analyzed using one-way repeated measures ANOVA, followed by Newman-Keuls multiple comparisons post-test (Prism 4 for Macintosh, GraphPad Software), or two-way ANOVA followed by Bonferroni post-test. Aqueous dynamics data are shown as box plots in figures and as mean ± S.D. in Table 1 and were compared using the *t* test function (two-tailed, homoscedastic) in Microsoft Excel 2004 for MAC.

RESULTS

To understand how bicarbonate may regulate aqueous flow, we performed assays of adenylyl cyclase activity on pig and human CB homogenates. In 1993, Mittag *et al.* (16) reported a bicarbonate-sensitive adenylyl cyclase activity in these tissues, and we sought to determine whether this was due to sAC expression. We assayed adenylyl cyclase activity in the presence or absence of bicarbonate and in the presence of the sAC-specific inhibitor KH7 (Fig. 1, *A* and *B*). In tissue homogenates, basal adenylyl cyclase activity will reflect contributions from both G protein-regulated transmembrane adenylyl cyclases (tmACs) and sAC, but the tmAC contribution will be unaffected by bicarbonate stimulation (22) or by KH7 inhibition (17). In porcine CB lysates, bicarbonate significantly stimulated (148 ± 26%, $n = 5$) and KH7 significantly diminished (61 ±

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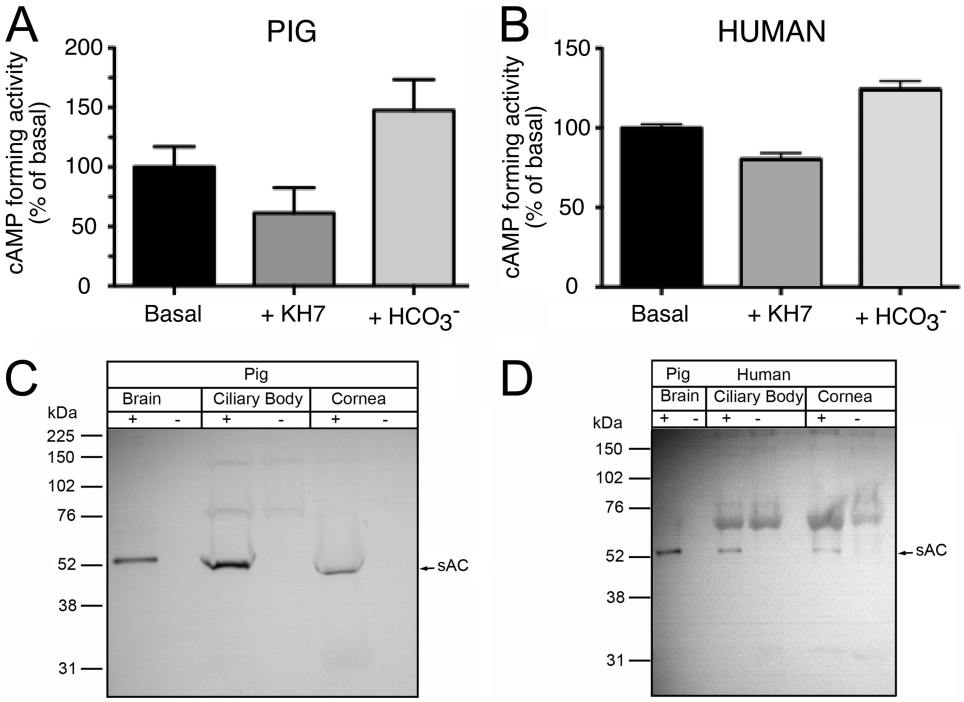

FIGURE 1. **Expression of sAC in pig and human CB.** *A* and *B*, assays of adenylyl cyclase activity were performed on pig (*A*) or human (*B*) CB lysates. Shown are values normalized to basal activity. Basal adenylyl cyclase activity was 12.81 ± 2.19 and 2.01 ± 0.11 pmol of cAMP mg protein^{-1} min^{-1} for pig and human CB, respectively. KH7 reduced basal adenylyl cyclase activity to 7.86 ± 2.72 and 1.62 ± 0.11 pmol of cAMP mg protein^{-1} min^{-1} for pig and human, respectively. Bicarbonate increased adenylyl cyclase activity to 18.90 ± 3.31 and 2.48 ± 0.12 pmol of cAMP mg protein^{-1} min^{-1} for pig and human respectively. Data are mean ± S.E. (*error bars*). *C* and *D*, for pig $n = 5$, for human $n = 9$. A ~50-kDA band corresponding to pig (*C*) or human (*D*) sAC was identified by immunoprecipitation and blot back from CB lysates. Pig brain and cornea (*C*) or pig brain and human cornea (*D*) were used as positive controls. Lanes marked − (*minus*) in *C* and *D* were negative controls in which antibody R37 was omitted during immunoprecipitation.

21%, $n = 5$) cAMP production relative to basal levels. Production of cAMP in human CB lysate responded in similar fashion (bicarbonate stimulation = 124 ± 5%, KH7 inhibition = 80 ± 4%, $n = 9$). To confirm that KH7 inhibited only sAC and not tmAC, we repeated the assay using porcine CB lysates stimulated by the tmAC-specific activator, forskolin, in the absence of bicarbonate. Whereas KH7 exhibited a dose-dependent effect on bicarbonate-stimulated adenylyl cyclase activity, forskolin stimulated activity was unaffected (Fig. 2). These data indicate the CB contains bicarbonate-sensitive sAC activity. To confirm that CB expresses sAC, we immunoprecipitated the protein from isolated pig or human CB using a specific monoclonal antibody (R37) as before (18, 23). A band of ~50 kDa was observed in Western blots of immunoprecipitates from CB using a biotinylated distinct, nonoverlapping sAC-specific monoclonal antibody (R21) (18, 23) as well as in positive controls including pig brain and pig or human cornea (Fig. 1, *C* and *D*). This band was absent in controls in which monoclonal antibody R37 was omitted and matched the size of the band in positive controls, including pig brain and pig or human cornea.

We next examined expression of sAC in the mouse eye with the goal of using mice for functional assays to determine the role of sAC in the CB. The small size of the mouse eye limited our ability to examine sAC expression by immunoprecipitation or enzymatic assays. However, we could identify sAC mRNA using RT-PCR of isolated mouse CB (Fig. 3*A*). Immunofluorescence staining of mouse eyes using biotinylated anti-sAC monoclonal antibody R21 identified strong expression in CB where sAC co-localized in NPE cells with Best2 and was also strongly expressed in CB stroma, but not pigment epithelium cells (Fig. 3, *B–D*). sAC expression was also noted in corneal endothelia and epithelia, inner retina, and retinal pigment epithelium (RPE) cells. sAC expression was absent or at levels below detection in the tissues comprising the conventional outflow pathway, TM and Schlemm's canal.

To determine further whether outflow tissues express sAC, we probed for sAC in lysates from CB and TM by immunoprecipitation (Fig. 4*A*). Whereas immunoprecipitation of porcine CB lysates consistently revealed strong expression of sAC in CB (Figs. 1, *C* and *D*, and 4*A*), no sAC was observed in immunoprecipitates of TM lysates (Fig. 4*A*). Next, we assayed isolated porcine TM for sAC by measuring KH7-sensitive adenylyl cyclase activity (Fig. 4*B*). Mn^{2+}-ATP supports much higher levels of sAC activity than Mg^{2+}-ATP; therefore, we measured adenylyl cyclase activity in TM in the presence of Mn^{2+}-ATP to perform the most sensitive assay for sAC. In the presence of 10 mM Mn^{2+} basal adenylyl cyclase activity in TM was 122.48 pmol of cAMP/mg of protein. KH7 significantly ($p < 0.001$) inhibited adenylyl cyclase activity in CB by nearly 50%. In contrast, KH7 did not significantly affect adenylyl cyclase activity in TM. Because we could not identify sAC in TM by immunofluorescence, immunoprecipitation, or enzymatic assays, we conclude that in contrast to CB, where sAC activity is highly expressed, sAC is either absent from or expressed at trace levels in TM.

To determine whether sAC plays a role in regulating aqueous flow, we injected mice with the sAC inhibitor KH7. As shown in Fig. 5*A* and Table 1, mice receiving KH7 exhibited an increase in IOP but not EVP, indicating that the effect of KH7 was spe-

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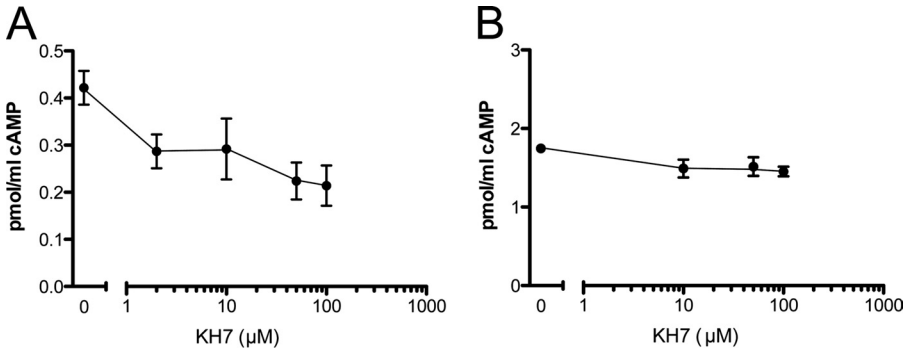

FIGURE 2. **Specificity of KH7 in human ciliary body lysate.** *A*, KH7 was added to human CB lysates at doses as indicated, and its effect on basal adenylyl cyclase activity was determined. *B*, to assess the effect of KH7 on tmAC, CB lysates were first stimulated with forskolin. KH7 had no significant effect on forskolin-stimulated adenylyl cyclase activity. Cyclase activity in the presence of forskolin alone was 1.746 ± 0.055. The results of KH7 treatment were 1.490 ± 0.11 (10 μM), 1.515 ± 0.12 (50 μM), and 1.452 ± 0.061 (100 μM). $n = 3$.

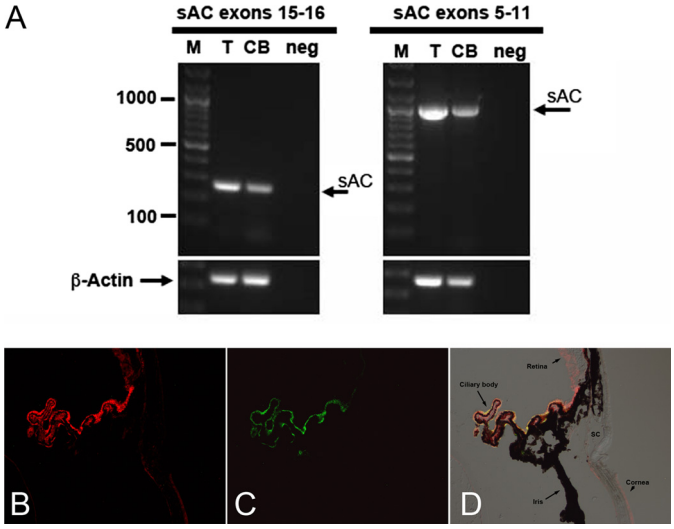

FIGURE 3. **sAC is expressed in CB but not drainage tissues.** *A–C*, mouse CB contain mRNA for sAC as evidenced by RT-PCR (*A*) using primer sets designed to span multiple exons covering two different regions of sAC. Bands matched those obtained from testis (*T*) and were absent in water controls (*neg*). Immunofluorescence staining for sAC (*B*) demonstrates that it is highly expressed in CB where it co-localizes with the NPE-specific protein Best2 (*C*). *D*, detailed examination of a merged fluorescence and differential interference contrast image demonstrates that sAC is highly expressed in CB where it is concentrated in NPE cells and stroma, but is not observed in pigment epithelium cells. Whereas sAC is also expressed in corneal endothelia and epithelia, and retina, no specific sAC staining was noted in iris, or drainage tissues in the region of Schlemm's canal (*SC*).

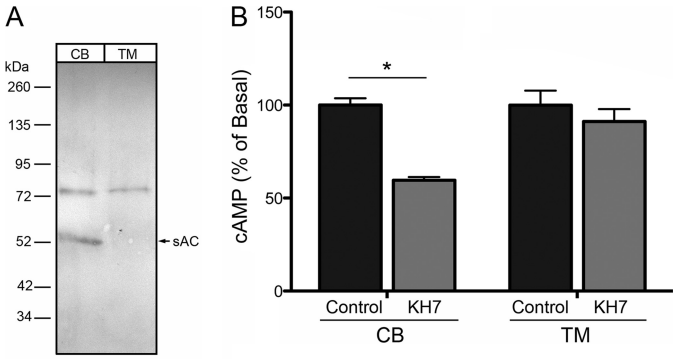

FIGURE 4. **sAC is not detected in TM.** *A*, sAC could be readily immunoprecipitated from porcine CB but was not present in immunoprecipitates from TM lysates. *B*, KH7 diminished basal adenylyl cyclase activity measured in porcine CB lysates incubated with varying doses of KH7, but not in TM lysates. *, $p < 0.001$.

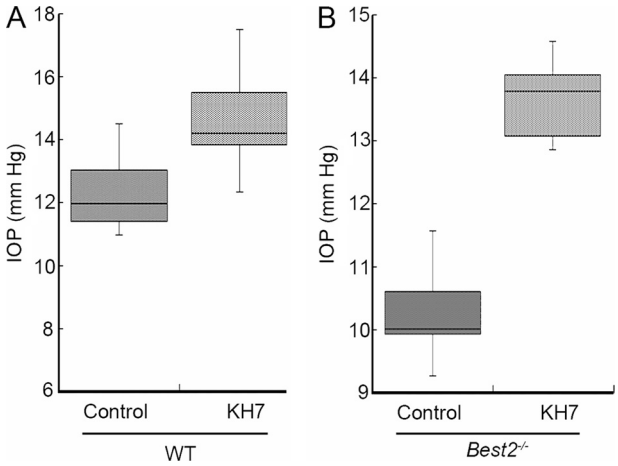

FIGURE 5. **Inhibition of sAC raises IOP.** IOP was measured in WT (*A*) or *Best2*$^{-/-}$ (*B*) mice injected with KH7 (5 μmol/kg) or vehicle alone (*Control*). IOP was significantly ($p < 0.01$) higher in KH7-treated mice compared with controls. Data are *box plots* in which 75% of measured values fall within the *box*, with remaining values in the range indicated by *error bars*. *Lines* in *boxes* represent median values. $n = 22$ control and 11 KH7 in *A*, $n = 5$ for KH7 and control in *B*.

cifically on aqueous flow. In KH7-treated mice IOP − EVP was elevated by an average of 42% over sham-injected controls. Similarly, KH7 treatment of *Best2*$^{-/-}$ mice (Fig. 5*B*) resulted in a significant ($p < 0.001$) increase in IOP (10.26 ± 0.87 for *Best2*$^{+/+}$ *versus* 13.70 ± 0.64 for *Best2*$^{-/-}$, mean ± S.D., $n = 5$) but not EVP. In *Best2*$^{-/-}$ mice KH7 treatment resulted in an increase in IOP − EVP of 46% over sham-injected controls.

To determine how IOP was increased we examined the effect of KH7 on F_a and C_t in WT mice. As shown in Fig. 6 and Table 1, administration of KH7 had no significant effect on F_a ($p = 0.77$), but caused an ~50% decrease in C_t ($p < 0.001$) compared with sham-injected controls. Using the measured parameters of IOP, EVP, F_a, and C_t, we derived the value for C_u. C_u was elevated by ~16% in KH7-treated mice *versus* controls, a difference that is likely within the range of our experimental error.

To ensure that the effects we observe were due to inhibition of sAC activity and not nonspecific effects of KH7, we next examined *Sacy*$^{tm1Lex/tm1Lex}$ mice (17, 24). These mice have been genetically altered to abolish the C1 catalytic domain of sAC. IOP in *Sacy*$^{tm1Lex/tm1Lex}$ mice was significantly ($p < 0.03$) ele-

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TABLE 1
Summary of effect of KH7 on aqueous humor dynamics in the WT mouse

Parameters	Control		KH7		
	Mean ± S.D.	*n*	Mean ± S.D.	*n*	*p*
IOP (mm Hg)	12.22 ± 1.08	22	14.60 ± 1.52	11	<0.005
EVP (mm Hg)	6.49 ± 0.30	12	6.45 ± .21	12	0.707
IOP − EVP (mm Hg)	5.73		8.15		
F_a (μl/min)	0.1996 ± .0404	8	0.2043 ± 0.332	10	0.797
C_t (μl*min^{-1}*mm Hg^{-2})	0.0105 ± .0021	8	0.0054 ± 0.0010	6	<0.001
C_u (μl/min)[a]	0.1377		0.1595		

[a] Derived from other values as described under "Experimental Procedures."

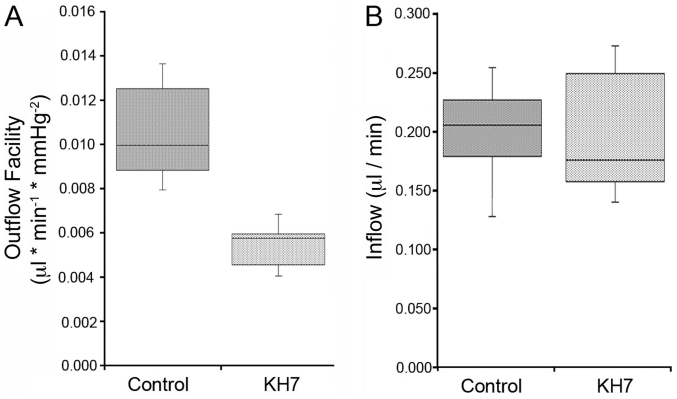

FIGURE 6. **Inhibition of sAC diminishes C_t but not F_a.** Aqueous dynamics studies were performed to assess the effect of KH7 C_t (*A*) and F_a (*B*) in WT mice. C_t was significantly ($p < 0.01$) lower in KH7-treated mice compared with controls, but F_a was unaltered. Data are *box plots* in which 75% of measured values fall within the *box*, with remaining values in the range indicated by *error bars*. *Lines* in *boxes* represent median values. The number of individual measurements made for each condition is as indicated in Table 1.

vated compared with *Sacy*$^{+/+}$ mice (Fig. 7*A*) (12.56 ± 0.56 *versus* 11.48 ± 0.79 for *Sacy*$^{tm1Lex/tm1Lex}$ *versus sAC*$^{+/+}$ mice, mean ± S.D., $n = 6$). Treatment of *Sacy*$^{tm1Lex/tm1Lex}$ mice with KH7 had little effect, bringing mean ± S.D. of IOP to 13.00 ± 0.49 ($p = 0.174$). We next examined C_t in KH7-treated *versus* untreated *Sacy*$^{tm1Lex/tm1Lex}$ mice. As shown in Fig. 7*B*, C_t in *Sacy*$^{tm1Lex/tm1Lex}$ mice was significantly ($p < 0.002$) lower than in *Sacy*$^{+/+}$ mice, and as expected, KH7 had no effect ($p = 0.75$).

DISCUSSION

The role of bicarbonate in the generation and regulation of aqueous flow is poorly understood. Although it is generally agreed that bicarbonate provides fuel for generating the Cl^- flux that drives generation of aqueous humor (5), regulation of drainage by the CB has, to date, remained in the realm of speculation (25). Here, we have shown that high levels of a bicarbonate-sensitive adenylyl cyclase activity are found in CB (Figs. 1, *A* and *B*, and 2). A portion of the basal adenylyl cyclase activity is inhibited by KH7 (Figs. 1, *A* and *B*, and 2), a highly specific inhibitor of sAC (17), and the enzyme was immunoprecipitated from pig and human ciliary body (Fig. 1, *C* and *D*). In mouse, mRNA for sAC was found in CB (Fig. 3*A*). Immunofluorescence experiments in mouse demonstrated high levels of sAC in CB, specifically in NPE and stromal cells (Fig. 3, *B* and *D*). However, sAC was not observed to be present in drainage tissues by immunofluorescence, immunoprecipitation, or enzymatic assays (Figs. 1, *C* and *D*, 2, *B–D*, and 4) although its expression in other tissues such as corneal endothelia (26, 27) and retinal ganglion cells (28), previously demonstrated to express sAC, was confirmed. Administration of KH7 to either WT or *Best2*$^{-/-}$ mice (Fig. 5) raised IOP independent of EVP; this effect was due entirely to a suppression of C_t (Fig. 5 and Table 1). *Sacy*$^{tm1Lex/tm1Lex}$, which lacks sAC activity, exhibited an increase in IOP and decreased C_t compared with *Sacy*$^{+/+}$ mice (Fig. 7). Furthermore, the specificity of KH7 effects in the mouse was confirmed by the absence of a KH7 effect on IOP or C_t on the *Sacy*$^{tm1Lex/tm1Lex}$ mouse (Fig. 7). Based on these data, we conclude that a pathway for acute regulation of C_t exists between the CB and drainage tissues and that this pathway responds to bicarbonate using the bicarbonate sensor sAC.

The idea that the CB secretes substances into the AH that can regulate C_t is not new. Escribano and Coca-Prados (29) demonstrated that ciliary epithelial cells produce neuropeptides, leading them to hypothesize that the CB may function as a neuroendocrine gland, regulating drainage by secreting neuropeptides or other substances into the AH (25, 29). However, to date, there have been no experimental data demonstrating that the CB can influence IOP by any mechanism other than changing F_a, which in effect is increasing pressure. Our prior studies using *Best2*$^{-/-}$ mice suggested that a communicative pathway exists between the CB and drainage tissues. In the eye, Best2 is expressed uniquely in the NPE (Fig. 3*B*) (11, 12). The effect of knocking out *Best2* was to decrease IOP despite an increase in F_a (11, 13). Measurement of aqueous dynamics in those mice demonstrated that both C_t and C_u were increased to more than compensate for the increase in F_a. However, the complexity of aqueous dynamics in these mice, and the possibility of compensation for the knock-out, did not allow us to rule out that enhanced drainage in *Best2*$^{-/-}$ mice could be due to something other than direct communication between the CB and drainage tissues.

Our first hint of a mechanism behind the effect of Best2 on drainage came from the effect of the carbonic anhydrase inhibitor brinzolamide on IOP in *Best2*$^{+/-}$ mice (11). Topical application of brinzolamide led to a greater decrease in IOP than was observed in *Best2*$^{+/+}$ mice, suggesting a role for bicarbonate in these effects. Indeed, we have since shown that Best2 functions as a critical bicarbonate channel in colon goblet cells (10), and it is likely that it functions similarly in NPE cells. This led us to examine the role of sAC in regulation of IOP. As shown in Figs. 5 and 6*A* and Table 1, inhibition of sAC increases IOP by decreasing C_t. Because sAC expression was below our limit of detection in drainage tissues (Figs. 2, *B–D*, and 4), we propose that 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. Based on these

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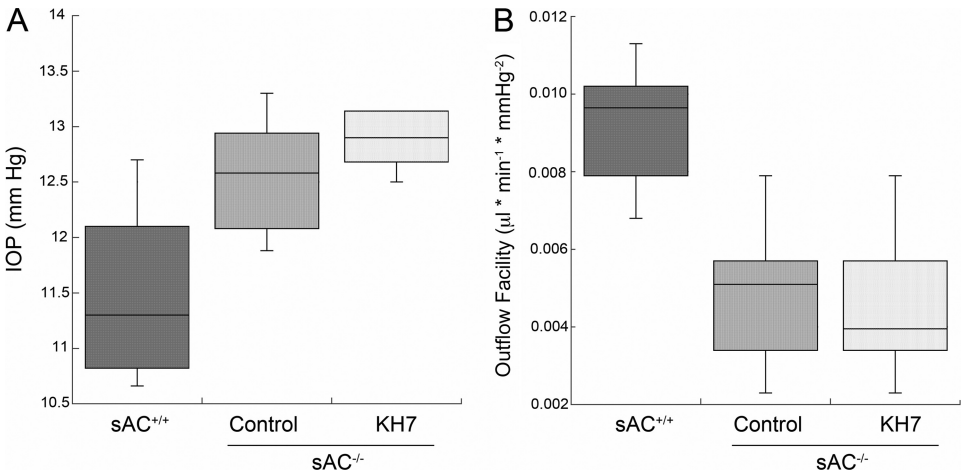

FIGURE 7. **IOP and C_t in *Sacy*$^{tm1Lex/tm1Lex}$ mice are unaffected by KH7.** *A*, IOP in *Sacy*$^{tm1Lex/tm1Lex}$ mice is elevated compared with *Sacy*$^{+/+}$ mice but unaffected by KH7. *B*, similarly, C_t, although diminished in *Sacy*$^{tm1Lex/tm1Lex}$ mice relative to *Sacy*$^{+/+}$ mice, is unaffected by KH7 in *Sacy*$^{tm1Lex/tm1Lex}$ mice. Data are *box plots* in which at least 75% of measured values fall within the box, with remaining values in the range indicated by *error bars*. *Lines* in *boxes* represent median values.

data, we propose that bicarbonate stimulates sAC activity in the NPE and fuels Cl^- uptake. When bicarbonate levels are high in NPE cells, F_a is high. Under these conditions, sAC is maximally stimulated, causing the NPE to secrete factors into the AH that cause C_t to increase to accommodate the increase in F_a. Future studies will clarify additional steps in this pathway and identify the specific messengers secreted into AH in response to sAC activity. Further characterization of this pathway represents a new avenue for the development of glaucoma therapeutics.

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