Physiology and Pharmacology

cAMP Stimulates Transepithelial Short-Circuit Current and Fluid Transport Across Porcine Ciliary Epithelium

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Purpose. To investigate the effects of cAMP on transepithelial electrical parameters and fluid transport across porcine ciliary epithelium.

Methods. Transepithelial electrical parameters were determined by mounting freshly isolated porcine ciliary epithelium in a modified Ussing chamber. Similarly, fluid movement across intact ciliary body was measured with a custom-made fluid flow chamber.

Results. Addition of 1, 10, and 100 μM 8-Br-cAMP (cAMP) to the aqueous side (nonpigmented ciliary epithelium, NPE) induced a sustained increase in short-circuit current (I_{sc}). Addition of niflumic acid (NFA) to the aqueous surface effectively blocked the cAMP-induced I_{sc} stimulation. The administration of cAMP to the stromal side (pigmented ciliary epithelium, PE) triggered a significant stimulation of I_{sc} only at 100 μM. No additive effect was observed with bilateral application of cAMP. Likewise, forskolin caused a significant stimulation of I_{sc} when applied to the aqueous side. Concomitantly, cAMP and forskolin increased fluid transport across porcine ciliary epithelium, and this stimulation was effectively inhibited by aqueous NFA. Depleting Cl^- in the bathing solution abolished the baseline I_{sc} and inhibited the subsequent stimulation by cAMP. Pretreatment with protein kinase A (PKA) blockers (H89/KT5720) significantly inhibited the cAMP- and forskolin-induced I_{sc} responses.

Conclusions. Our results suggest that cAMP triggers a sustained stimulation of Cl^- and fluid transport across porcine ciliary epithelium; Cl^- channels in the NPE cells are potentially a cellular site for this PKA-sensitive cAMP-mediated response.

Keywords: chloride channel, ciliary epithelium, cyclic adenosine monophosphate, short-circuit current, fluid flow

Elevated intraocular pressure (IOP) is an important risk factor for glaucoma, leading to irreversible optic neuropathy. Lowering IOP is the only clinical intervention proven to be effective in delaying the onset and slowing the progression of vision loss.[1,2] The level of IOP reflects a balance between aqueous humor secretion and outflow facility. Aqueous humor is secreted by the ciliary epithelium comprising the pigmented ciliary epithelium (PE) and nonpigmented ciliary epithelium (NPE). The secretion of aqueous humor is driven primarily by a net Cl^- transport across this bilayered epithelium in three major transport steps: (1) Cl^- uptake by PE cells; (2) Cl^- diffusion from PE to NPE cells through gap junctions; and (3) Cl^- efflux by NPE cells into the posterior chamber.[3–5] Among these steps, Cl^- efflux by NPE cells likely limits the rate of aqueous humor secretion. We have previously demonstrated that the addition of Cl^- channel blockers to the excised ciliary epithelium causes a simultaneous inhibition of short-circuit current (I_{sc}) and net Cl^- transport by 80% to 90%.[4,6]

Many cellular signaling cascades are involved in the regulation of aqueous humor formation. Among them, cyclic adenosine monophosphate (cAMP) has long been suggested to play an important role in the regulation of aqueous inflow and IOP, but its physiological significance remains controversial. In the ciliary epithelium, beta-adrenergic receptors have been identified in both animals and humans.[7,8] Binding of adrenergic agonists to these membrane receptors stimulates adenylate cyclase, leading to cAMP formation. It has been shown that the administration of beta-adrenergic agonists and forskolin (a direct activator of adenylate cyclase) decreases the IOP.[9–11] Nevertheless, beta-adrenergic antagonists (beta-blockers) have been used clinically to lower IOP primarily by decreasing the rate of aqueous humor formation.[12] It remains unclear why adrenergic agonists and antagonists produce parallel ocular hypotensive responses and whether beta-blockers act through a cAMP-dependent pathway.[13]

Based upon the previous results obtained from bovine preparations, cAMP is shown to inhibit net Cl^- transport across the excised ciliary epithelium.[14] Addition of forskolin and 8-bromo-cAMP to the ciliary epithelium decreases the net Cl^- secretion into the eye, suggesting a cAMP-inhibitable Cl^- transport mechanism in bovine ciliary epithelium.[14] The inhibitory effect of cAMP is possibly mediated by the regulation of gap junction permeability between PE and NPE cells.[15] However, it has been shown that the addition of cAMP induces a significant increase in Cl^- secretion in porcine eyes.[16] These opposing results hinder the understanding of the precise functional significance of cAMP in regulating aqueous humor secretion. Significant species variations in the transport

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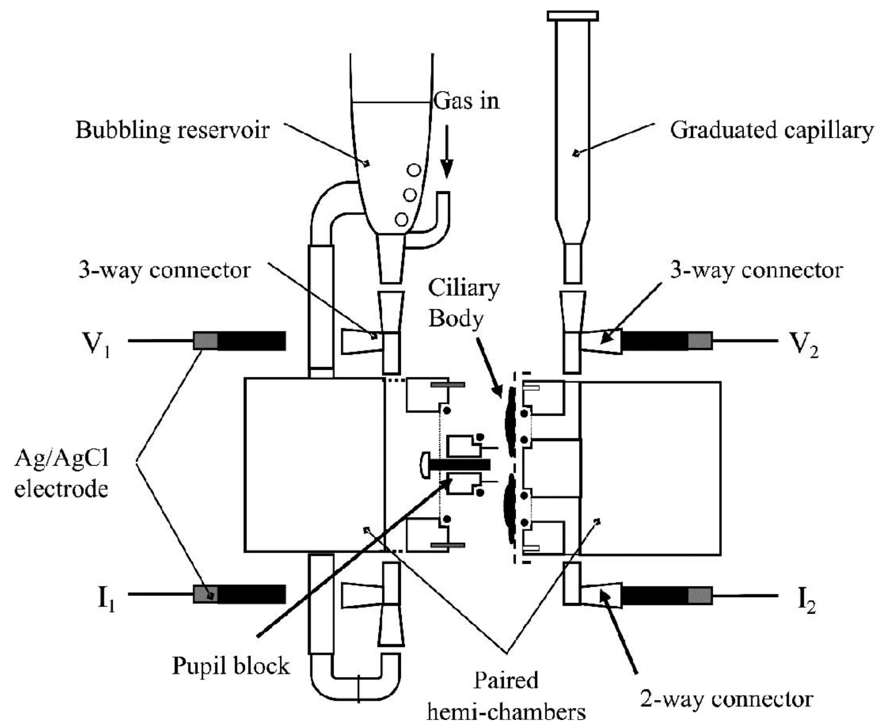

FIGURE 1. Schematic diagram illustrating the configuration of a fluid flow chamber.

mechanisms across the ciliary epithelium have been reported; therefore the full implications of these results for humans are yet to be determined.[6,17] The aim of this study was to investigate how cAMP affects aqueous humor secretion in the porcine eye. This question is addressed by using physiological approaches to study the effects of cAMP on transepithelial electrical measurements and fluid movement across isolated ciliary body preparations.

METHODS

Tissue Preparation for Electrical and Fluid Flow Measurements

Freshly enucleated porcine eyes were obtained from a local abattoir. For Ussing chamber experiments, a sector of ciliary body/epithelium was excised,[4,6] and for fluid flow chamber measurements, an intact annulus ring of ciliary body with iris attached was dissected and mounted to the chamber.[18,19]

Modified Ussing Chamber

A custom-made modified Ussing chamber used in the experiment was identical to that used in a previous study.[6] The isolated ciliary body preparation was mounted into the chamber with an exposed area of 0.10 cm^2. Bathing solution (15 mL) was filled to each side of the chamber, and 95% O_2-5% CO_2 was delivered to the solution throughout the experiment conducted at 23°C to 25°C.

Transepithelial electrical parameters including the spontaneous transepithelial electrical potential difference (PD), I_{sc}, and tissue resistance (R_t) were monitored continuously with a dual voltage current clamp unit (DVC-1000; World Precision Instruments, Sarasota, FL, USA). Two pairs of NaCl polyacrylamide gel (0.9%) filled with Ag/AgCl electrodes (World Precision Instruments) were used to measure the PD and pass a known current across the tissue preparation, respectively.

Fluid Flow Chamber

The fluid flow chamber was identical to the ones used in previous studies.[18,19] As shown in Figure 1, minor modifications were made with an additional pair of electrodes that was connected to the chamber for the measurement of R_t. Similar to the procedure with the Ussing chamber, the ciliary body preparation was mounted between two hemi-chambers; one hemi-chamber was connected to an open bubbling reservoir, facilitating drug administration to the chamber. The other hemi-chamber was connected to a 25-μL capillary tube with graduations of 0.25 μL. A complete annulus ring of the isolated porcine ciliary body was mounted into the chamber (exposed area was 0.78 cm^2) with the aqueous and stromal surfaces of the ciliary epithelium facing each hemi-chamber, respectively. Bathing solution was filled on both sides of the chamber and bubbled with 95% O_2-5% CO_2 throughout the experiment.

As shown in Figure 1, measurement of spontaneous fluid flow was determined by the changes in water level in the capillary tube. Drug administration was achieved unilaterally only on the hemi-chamber connecting to the bubbling reservoir.[19] To minimize pressure differences across the tissue preparation, the water level of the capillary was adjusted to $<$ 5-mm difference from the water level of the bubbling reservoir during experiments. The changes in capillary volume were recorded at 15-minute intervals and converted to the rate of fluid flow across the preparation. Recording of electrical parameters was similar to that for the Ussing chamber experiments described above.

Intracellular cAMP Level in Porcine Ciliary Epithelium

As in our previous studies,[15,20] native porcine PE and NPE cells were harvested from freshly isolated ciliary body pieces by incubating with 0.25% HyClone trypsin (Thermo Scientific, Burlington, ONT, Canada) for 30 minutes at 37°C. The extracted cells were incubated for 48 hours in cell culture

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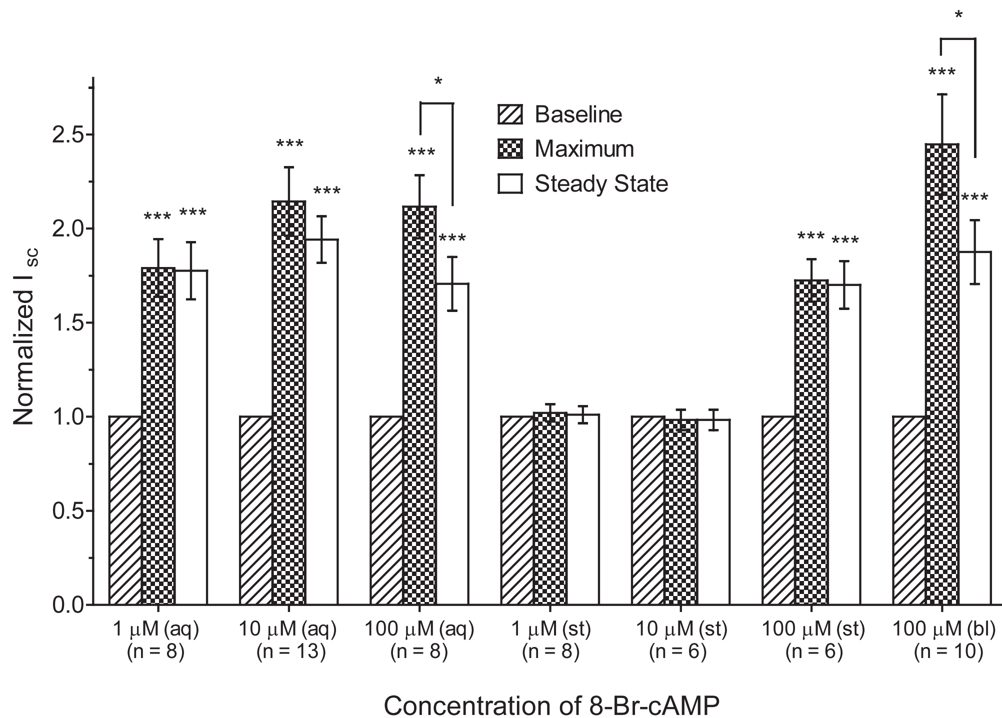

FIGURE 2. Effects of cAMP on I_{sc}. 8-Br-cAMP was administered to the aqueous (aq) or stromal (st) side of the porcine ciliary body or bilaterally (bl). 1-way repeated measures ANOVA, $^{***}P < 0.001$ compared with baseline, $^{*}P < 0.05$.

medium containing high-glucose HyClone Dulbecco's modified Eagle's medium (DMEM;Thermo Scientific), 10% fetal bovine serum (GIBCO, Thermo Scientific), and 1% penicillin-streptomycin (GIBCO, Thermo Scientific). The cells were then resuspended and incubated with cell culture medium with or without forskolin. Thereafter, the cells were lysed with 0.1 M HCl before centrifugation. The supernatants were used for protein and cAMP concentration assays.

The amount of protein in each sample was quantified using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), and the intracellular cAMP level was determined using a cAMP Direct Immunoassay Kit (ab65355; Abcam, Cambridge, MA, USA) following the procedures suggested by the manufacturer. The samples were assayed in duplicate for both assays, and the intracellular cAMP concentrations were expressed in pmol/mg by dividing the cAMP concentration with protein concentration.

Bathing Solutions and Pharmacologic Agents

Bathing solution used in the chamber experiments was HEPES buffered Ringer's solution that comprised (in mM) NaCl 113.0, KCl 4.56, $NaHCO_3$ 21.0, $MgSO_4$ 0.6, D-glucose 7.5, reduced glutathione 1.0, Na_2HPO_4 1.0, HEPES 10.0, and $CaCl_2$ 1.4. The osmolality was adjusted to 290 mOsm/kg with D-mannitol, and pH was adjusted to 7.4. Low Cl^- bathing solution was prepared by replacing sodium chloride with an equimolar amount of sodium cyclamate.

8-Br-cAMP, forskolin, IBMX (3-isobutyl-1-methylxanthine), niflumic acid (NFA), KT5720, and H89 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). 8-Br-cAMP and H89 were dissolved in distilled water. Forskolin, IBMX, and NFA were dissolved in dimethyl sulfoxide (DMSO), whereas KT5720 was dissolved in methanol. The final concentration of DMSO and methanol was adjusted to 0.1% or below.

Statistical Analysis

All data were expressed as mean ± SEM. GraphPad Prism 5 (San Diego, CA, USA) was used for statistical analysis. 1-way ANOVA or *t*-test as paired or unpaired data was performed for data comparison. A *P* value less than 0.05 was regarded as statistically significant.

RESULTS

Baseline Transepithelial Electrical Parameters

Baseline transepithelial PD, I_{sc}, and R_t recorded from the Ussing chamber were 0.89 ± 0.02 mV (aqueous side negative relative to the stromal side), 11.2 ± 0.3 $\mu A \cdot cm^{-2}$, and 82 ± 2 $\Omega \cdot cm^2$, respectively ($n = 167$).

Stimulation of I_{sc} by cAMP and Forskolin

The effect of 8-Br-cAMP (a cell-permeable cAMP analogue that is more resistant to phosphodiesterases) on I_{sc} is summarized in Figure 2 and Table 1. All three concentrations, 1, 10, and 100 μM 8-Br-cAMP (cAMP), elicited a sustained stimulation of I_{sc} when administered to the aqueous side by 78%, 94%, and 71%, respectively. No significant difference was observed among these three groups. At 100 μM, the addition of cAMP to the aqueous side triggered a biphasic response with a rapid, transient 2-fold I_{sc} stimulation before plateauing at steady state after 60 to 90 minutes ($P < 0.05$). In addition, cAMP acted more effectively when applied to the aqueous surface compared to the stromal surface. The stimulation of I_{sc} was observed only by 100 μM stromal cAMP with no biphasic response elicited. For the bilateral administration of 100 μM cAMP, a transient I_{sc} stimulation peak was observed before reaching a steady state, similar to aqueous application. Bilateral

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TABLE 1. Effects of 8-Br-cAMP and Forskolin on I_{sc}

		I_{sc}, $\mu A \cdot cm^{-2}$		
			Drug Treated	
Condition	n	Baseline	Maximum	Steady State
8-Br-cAMP				
1 μM (aq)	8	10.6 ± 1.4	18.3 ± 2.2	18.1 ± 2.1
10 μM (aq)	13	10.5 ± 1.0	21.0 ± 1.1	19.4 ± 1.2
100 μM (aq)	8	9.9 ± 1.6	21.1 ± 3.8	17.5 ± 3.4
1 μM (st)	8	8.7 ± 1.0	8.8 ± 0.9	8.7 ± 0.9
10 μM (st)	6	9.4 ± 0.5	9.3 ± 0.9	9.3 ± 0.9
100 μM (st)	6	9.4 ± 1.0	16.0 ± 1.5	15.7 ± 1.4
100 μM (bl)	10	11.2 ± 0.8	26.1 ± 1.9	20.3 ± 1.5
Forskolin				
500 nM (aq)	5	13.5 ± 1.3	23.3 ± 3.1	23.3 ± 3.1
1 μM (aq)	9	10.6 ± 1.9	23.9 ± 4.3	23.7 ± 4.2
10 μM (aq)	8	10.1 ± 1.3	21.5 ± 2.8	21.2 ± 2.6
10 μM (st)	6	11.6 ± 0.8	20.6 ± 2.0	19.6 ± 2.2

Data are expressed as the mean ± SEM. n = number of experiments. (aq), applied to aqueous side; (st), applied to stromal side; (bl), applied bilaterally (aqueous and stromal sides).

application of cAMP did not produce an additive effect, as the steady-state stimulation of I_{sc} induced by bilateral and aqueous-side application was comparable ($P > 0.05$, unpaired t-test). Additionally, aqueous administration of 1 mM NFA (a Cl^- channel blocker) significantly blocked the cAMP-induced I_{sc} stimulation (Fig. 3A). To substantiate the contribution of Cl^- secretion in cAMP-mediated I_{sc} stimulation, we studied the effect of low Cl^- bath on I_{sc} stimulation induced by cAMP. As shown in Figure 3B and Table 2, substituting the bathing Cl^- with cyclamate abolished the baseline I_{sc}. Subsequent addition of cAMP to the aqueous or stromal side did not trigger a significant increase in I_{sc} across porcine ciliary body.

Similar to cAMP, aqueous application of forskolin triggered a significant increase in I_{sc} (Fig. 4; Table 1). No biphasic stimulation was observed. Likewise, aqueous-side application of forskolin was shown to act more effectively than stromal administration ($P < 0.05$). Furthermore, addition of 1 mM NFA to the aqueous surface effectively inhibited the stimulation of I_{sc} by aqueous forskolin ($n = 3$, $P < 0.001$).

However, IBMX (a nonspecific inhibitor of cAMP phosphodiesterases that prevents the degradation of cAMP) when added to the aqueous surface at 100 μM could not trigger a significant effect on I_{sc} (Fig. 5). Subsequent aqueous-side application of 1 μM forskolin caused a stimulation that was comparable to that without IBMX pretreatment ($P > 0.05$, unpaired t-test).

Effects of cAMP and Forskolin on Transepithelial Fluid Movement

To relate the effects of cAMP and forskolin on I_{sc} to fluid secretion, transepithelial fluid flow and electrical parameters were measured simultaneously using the fluid flow chamber. Fluid transport was found to be in the stromal-to-aqueous direction. The average fluid flow rate in the 1-hour period prior to first drug administration was taken as the baseline fluid flow rate. Electrical parameters just before the administration of cAMP/forskolin were taken as the baseline values. As shown in Figure 6, administration of 10 μM cAMP to the aqueous side increased the fluid flow rate significantly from the baseline value of 1.8 ± 0.3 μL/h per preparation to a maximum of 4.0 ± 0.7 μL/h per preparation (Fig. 6A), and this effect lasted for at least 2 hours. Similar to the results obtained from the modified Ussing chamber, a significant stimulation of PD and I_{sc} was observed concomitantly with the fluid flow rate (Figs. 6B, 6D, respectively). Tissue resistance remained stable throughout the experimental period (Fig. 6C).

Similarly to cAMP, aqueous administration of 1 μM forskolin triggered a sustained increase in fluid flow rate from 2.4 ± 0.3 μL/h per preparation to 4.6 ± 0.7 μL/h per preparation ($n = 7$, Fig. 7A). Concomitant with the stimulation of fluid flow, forskolin also significantly hyperpolarized PD and increased I_{sc} (Figs. 7B, 7D, respectively). The forskolin-induced stimulatory effect on I_{sc} was comparable to the results obtained from the modified Ussing chamber (Fig. 4). However, R_t was found to be statistically significant from baseline value although the actual reduction of R_t was only 3.8% (Fig. 7C). Subsequently, 1 mM NFA was administered to the aqueous side of the tissue preparation 2 hours after forskolin treatment. Niflumic acid significantly blocked the forskolin-stimulated fluid flow ($n = 6$, $P < 0.001$) and PD ($P < 0.001$). Fluid flow and PD were found to be 1.5 ± 0.0 μL/h per preparation and 0.05 ± 0.04 mV (aqueous side negative), respectively, after the administration of NFA.

Stimulation of Endogenous cAMP Production by Forskolin

The effect of forskolin on intracellular cAMP content of porcine ciliary epithelial cells was studied. Exposing the porcine ciliary epithelial cells to 1 μM forskolin for 10, 20, and 30 minutes significantly increased intracellular cAMP level by ~50% to 60% (Fig. 8).

Effects of PKA Blockers on cAMP- and Forskolin-Stimulated I_{sc}

The tissue preparation was pretreated with protein kinase A (PKA) inhibitors (H89 or KT5720) bilaterally for 30 minutes. Both H89 (50 μM, $n = 14$, $P > 0.05$) and KT5720 (5 μM, $n = 8$, $P > 0.05$) had no effects on baseline I_{sc}. Figure 9 and Table 3 summarize the inhibitory effects of H89 and KT5720 on cAMP- or forskolin-induced I_{sc} stimulation. H89 significantly inhibited the I_{sc} stimulation induced by cAMP and forskolin. Likewise, KT5720 effectively inhibited the cAMP-induced I_{sc} stimulation by ~60% (Fig. 9; Table 3).

DISCUSSION

Our results demonstrated for the first time that cAMP and forskolin stimulated concomitant transepithelial electrical parameters and fluid movement across freshly excised porcine ciliary epithelium, supporting the crucial role of cAMP in regulating the rate of aqueous humor secretion. The stimulation was prevented by either low bath Cl^- or administration of NFA to the aqueous side, indicating that cAMP acted by stimulating transepithelial Cl^- secretion in ciliary epithelium. Protein kinase A blockers partially inhibited the I_{sc} stimulation by cAMP and forskolin.

Stimulation of I_{sc} by cAMP and Forskolin

Administration of cAMP to the aqueous side at different concentrations (1, 10, and 100 μM) elicited a sustained stimulation of I_{sc}. At 100 μM, aqueous cAMP induced a biphasic response, namely, a transient peak followed by plateau stimulation. Cyclic AMP worked less effectively when added to the stromal side, as only the highest concentration of stromal cAMP (100 μM) triggered a significant stimulation. Our results were consistent with a previous porcine study in which

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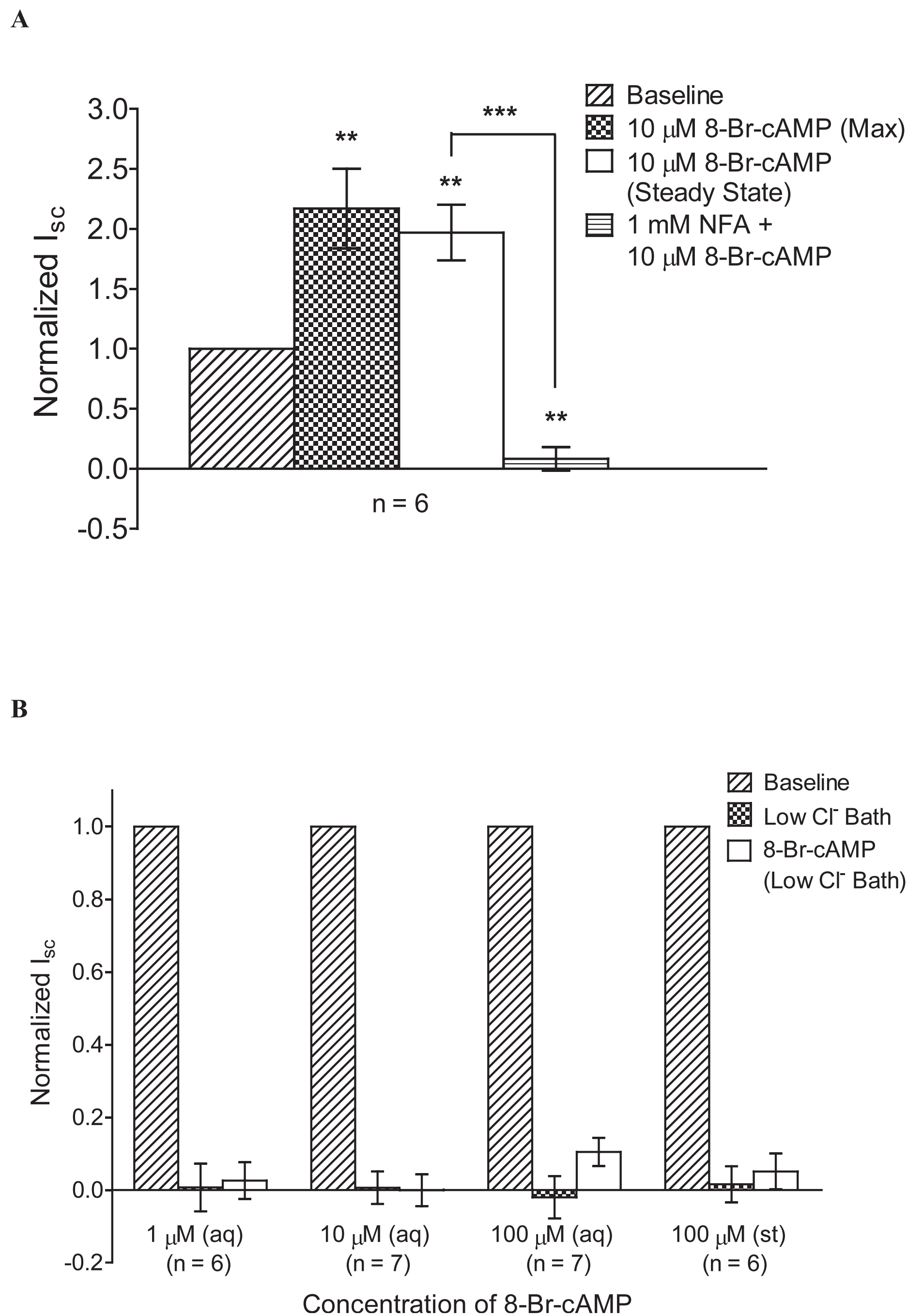

FIGURE 3. Effects of (A) NFA and (B) low bathing Cl^- on cAMP-induced I_{sc} stimulation. (A) cAMP and NFA were applied to the aqueous side of the ciliary body. The I_{sc} under baseline, cAMP-treated, and NFA+cAMP-treated conditions was found to be 11.4 ± 1.8, 20.4 ± 1.7, and 1.5 ± 1.3 $\mu A \cdot cm^{-2}$, respectively. (B) Bathing Cl^- concentration was reduced from 120 to 7 mM. cAMP was then administered to the aqueous (aq) or stromal (st) side of the porcine ciliary body. Comparisons were made using 1-way repeated measures ANOVA, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with baseline unless otherwise stated.

an increase in I_{sc} was shown with aqueous 10 μM to 1 mM cAMP.[16] Our results indicate that cAMP works at a lower concentration (1 μM), which is possibly more pertinent to physiological conditions.[21] In addition to the cAMP analogue, we studied the effects of forskolin, a direct activator of adenylate cyclase. Our results showed that forskolin stimulated intracellular cAMP production in porcine ciliary epithelial cells and elicited a sustained increase in I_{sc}, suggesting intact adenylate cyclase function in ciliary epithelium. As with cAMP, aqueous application of forskolin was more effective in stimulating I_{sc} compared with stromal application. On the other hand, IBMX could not trigger a significant response on baseline I_{sc}, indicating a low basal endogenous cAMP level in ciliary epithelium.[22] This is supported by the previous finding that IBMX did not affect the basal cAMP level in bovine ciliary epithelium.[23]

Likely, cAMP may exert multiple effects on both PE and NPE cells. Previous studies have shown that adenylate cyclase activity is higher in NPE cells than in PE cells.[24,25] This is consistent with our findings that both cAMP and forskolin

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TABLE 2. Effects of cAMP on I_{sc} in Low Bathing Cl⁻ Conditions

		I_{sc}, μA·cm⁻²		
8-Br-cAMP	*n*	Baseline	Low Cl⁻	8-Br-cAMP Administered, Low Cl⁻
1 μM (aq)	6	13.5 ± 1.3	−0.2 ± 1.0	0.2 ± 0.8
10 μM (aq)	7	13.2 ± 2.0	−0.1 ± 0.5	−0.2 ± 0.5
100 μM (aq)	7	11.0 ± 2.0	−0.2 ± 0.6	1.0 ± 0.4
100 μM (st)	6	11.6 ± 1.4	0.5 ± 0.7	0.9 ± 0.7

Data are expressed as the mean ± SEM. *n* = number of experiments. (aq), applied to aqueous side; (st), applied to stromal side.

trigger a larger stimulation of I_{sc} when added to the aqueous surface, suggesting that the major cellular target site(s) of cAMP may be located in NPE cells. This was supported by the evidence that no apparent additive stimulatory effect was observed between aqueous and bilateral administration of cAMP. On the other hand, stromal application of cAMP could trigger a stimulatory effect only at high concentration, and the magnitude of this I_{sc} stimulation was similar to that triggered by low concentration of cAMP administered to the aqueous surface. Besides, we realized that the latency of the response was significantly longer when cAMP was added to the stromal side (14 minutes) compared to the aqueous side (4 minutes). This may be caused by the presence of diffusional barrier in ciliary stroma lying near the basolateral membrane of PE cells, potentially hindering cAMP from reaching the target site(s) in the ciliary epithelium.

Concomitant Stimulation of Fluid Transport by cAMP and Forskolin

Our results demonstrate a stromal-to-aqueous fluid transport across porcine ciliary body, similar to that reported previously in pig[19] and cow.[26] Our measured baseline fluid flow rate was also comparable to that reported previously.[18,19] To the best of our knowledge, this is the first report of a direct cAMP-mediated fluid flow measured concomitantly with a stimulation of I_{sc}. Consistent with electrical measurements, our results show that aqueous administration of cAMP or forskolin elicited a significant increase in fluid movement across porcine ciliary body. Given that the effects of cAMP and forskolin on both electrical measurements and fluid transfer are similar and comparable, we studied the effect of NFA on forskolin-induced increase in fluid flow. Administration of aqueous NFA effectively blocked the forskolin-induced stimulation of both transepithelial fluid flow and I_{sc} simultaneously. These results strongly support the notion that cAMP stimulates the rate of aqueous humor secretion in porcine eyes. Besides, simultaneous and proportional changes in I_{sc} and fluid flow rate were observed in our preparations, suggesting that I_{sc} may be a good indicator for monitoring the changes in aqueous humor secretion.[18] Therefore, most of our subsequent experiments were conducted using the modified Ussing chamber because firstly, the preparation used was smaller in size and easier to handle, and secondly, it enabled the bilateral administration of drug, whereas the fluid flow chamber allowed only unilateral administration.

Potential Sites of cAMP's Action

Based upon previous findings, the NPE-cell Cl⁻ channel is a potential target of cAMP.[27] It is believed to be responsible for Cl⁻ efflux into the posterior chamber and has been suggested to be the rate-limiting step of transepithelial Cl⁻ transport.[28,29] Our results showed that cAMP triggered a significant increase in I_{sc}, and this cAMP-stimulated response was inhibited under low bathing Cl⁻ condition and by aqueous NFA, indicating that the target site of cAMP was likely located on the basolateral membrane of porcine NPE cells. It is plausible that the stimulatory effect of cAMP is mediated by upregulating Cl⁻ channels in the NPE cells,[30,31] leading to an increase in Cl⁻

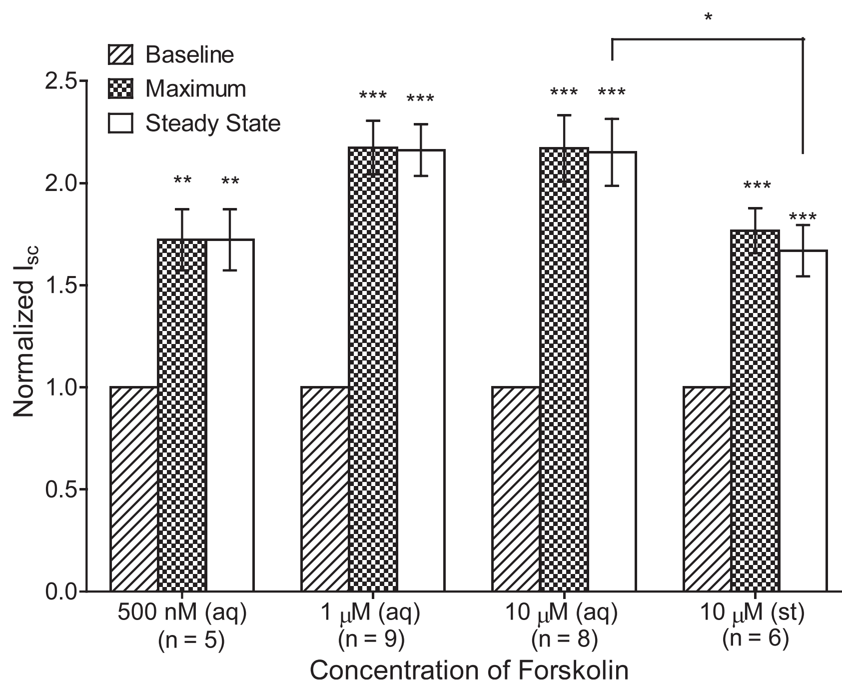

FIGURE 4. Effects of forskolin on I_{sc} across porcine ciliary body. Forskolin was applied to either the aqueous (aq) or stromal (st) side of the tissue preparation. 1-way repeated measures ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with baseline unless otherwise stated.

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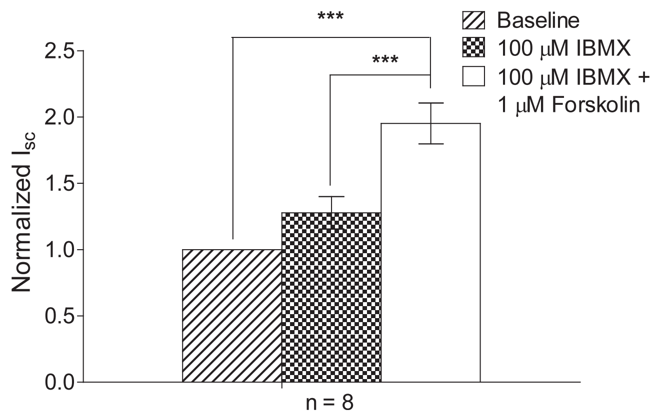

Figure 5. Sequential effects of IBMX and forskolin on I_{sc}. IBMX and forskolin were both applied to the aqueous side of the tissue preparation. 1-way repeated measures ANOVA, $^{***}P < 0.001$. The I_{sc} under baseline, IBMX-treated, and forskolin+IBMX-treated conditions was found to be 10.7 ± 1.3, 13.9 ± 2.4, and 21.0 ± 3.1 $\mu A \cdot cm^{-2}$, respectively.

efflux into the posterior chamber. Further investigations may be needed to verify this by monitoring the activities of Cl^- channels and intracellular Cl^- concentration in NPE cells upon cAMP stimulation.

Involvement of PKA Signaling Pathway

Our results demonstrated that both H89 and KT5720 inhibited cAMP-induced I_{sc}, indicating that the PKA signaling pathway is involved in the cAMP-mediated transepithelial ion transport and aqueous humor formation. However, the inhibition by PKA blockers was not complete but only partial, ranging from 50% to 60%. One of the possibilities was that in transepithelial measurements, the concentration of PKA blockers reaching the epithelial surface was not sufficient to inhibit the PKA pathway completely. It has been reported that H89 has limited cell membrane permeability[32]; the presence of other tissues such as the stroma and vitreous humor may retard the diffusion of drug in reaching its action sites. Another possibility was that there might be several cellular targets of PKA phosphorylation mediated by cAMP, each of these working differently in contributing to the resultant response observed. In addition, since we have only studied the effects of PKA blockers on I_{sc} stimulation triggered by cAMP, we could not exclude the possibility of cross-talk among different signaling pathway(s), for example, protein kinase G (PKG), which has been shown to be involved in the regulation of aqueous humor secretion.[33]

Species Variation

Several animal models have been used for the study of aqueous humor secretion; however, significant species variations in the

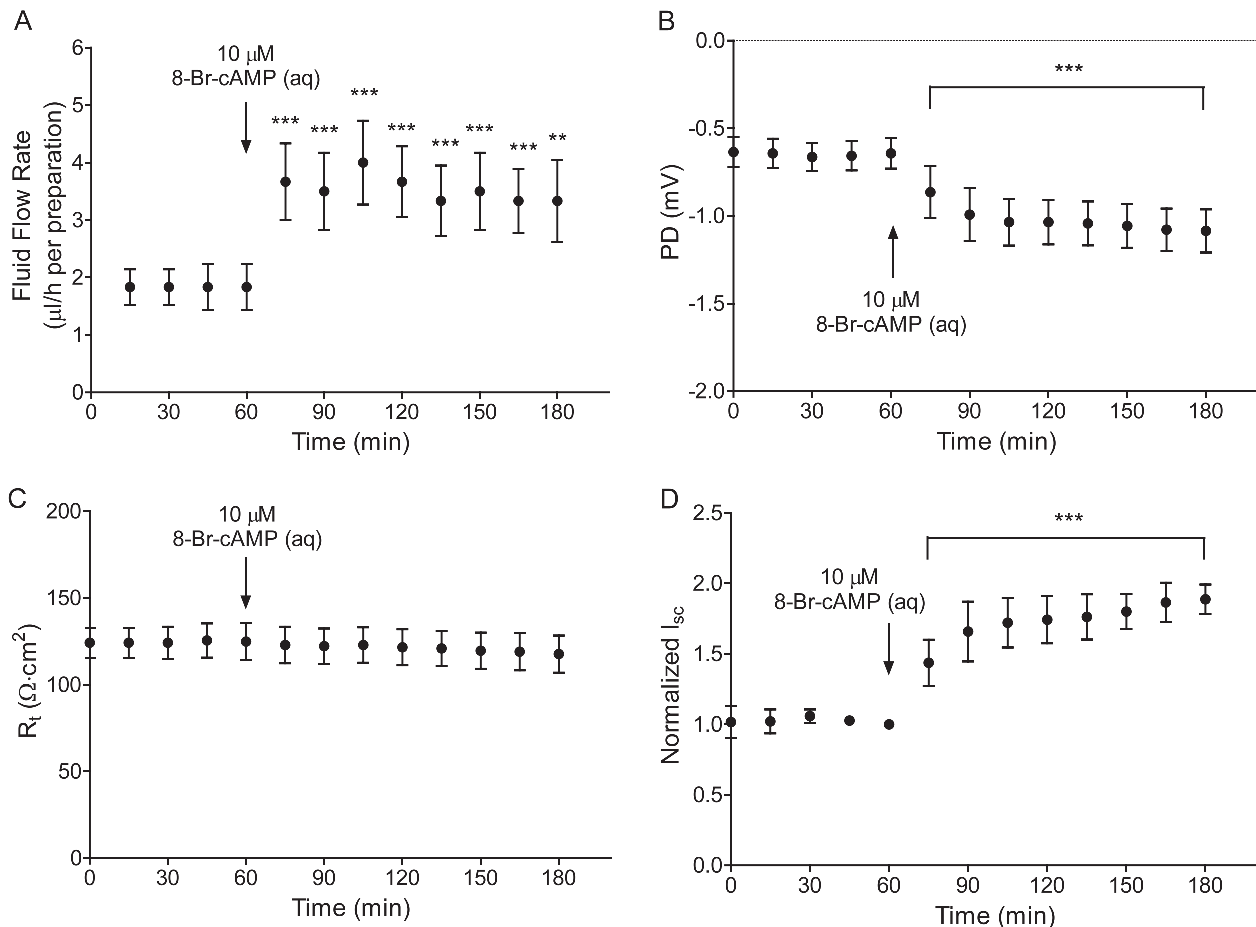

Figure 6. Effects of cAMP on (**A**) fluid flow, (**B**) PD, (**C**) R_t, and (**D**) I_{sc} across porcine ciliary epithelium using fluid flow chamber. Data were plotted as the mean ± SEM. Fluid flow, PD, R_t, and I_{sc} were monitored simultaneously. cAMP was administered to the aqueous (aq) side of the preparation. Average fluid flow rate in the 1-hour period prior to cAMP administration was regarded as the baseline. PD and I_{sc} right before the cAMP administration was taken as the baseline. I_{sc} was normalized to baseline I_{sc} ($n = 6$, 1-way repeated measures ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with baseline).

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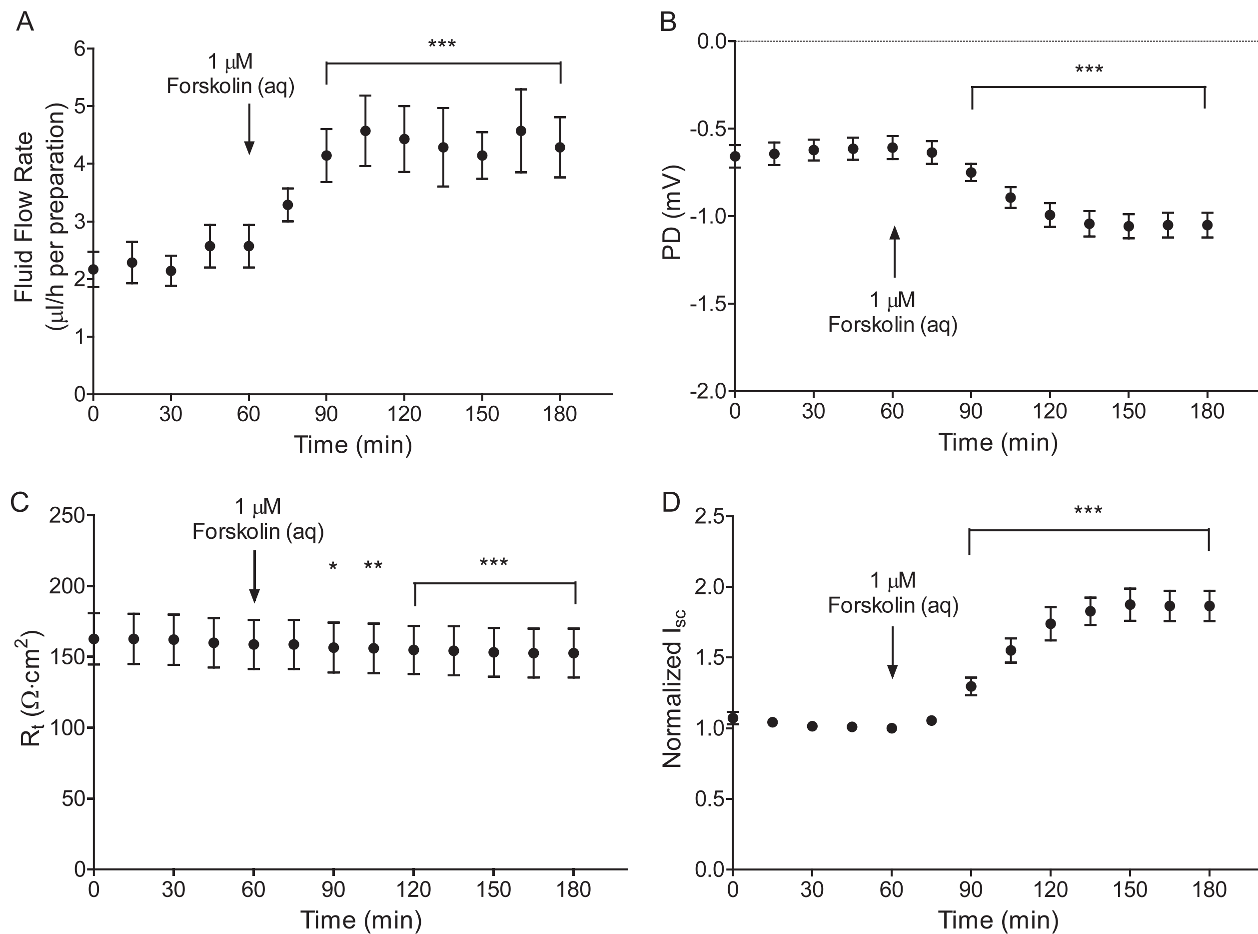

Figure 7. Effects of forskolin on (**A**) fluid flow, (**B**) PD, (**C**) R_t, and (**D**) I_{sc} across porcine ciliary epithelium using fluid flow chamber. Data points were plotted with mean ± SEM. Fluid flow, PD, R_t, and I_{sc} were monitored simultaneously. Forskolin was administered to the aqueous (aq) side of the ciliary body. Average fluid flow rate of the 1-hour period prior to forskolin administration was regarded as the baseline. PD and I_{sc} just before the administration of forskolin were regarded as the baseline. I_{sc} was normalized to baseline I_{sc} ($n = 7$, 1-way repeated measures ANOVA, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with baseline).

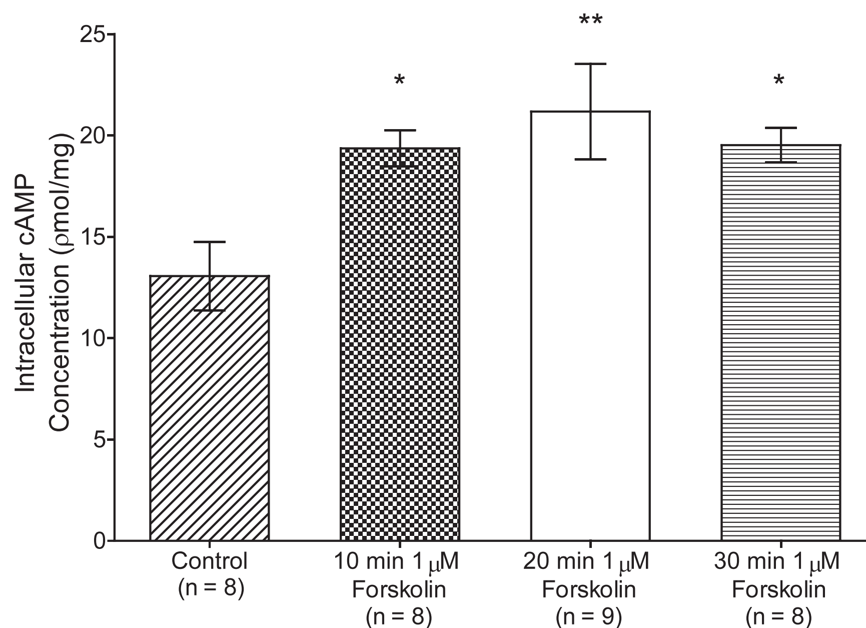

Figure 8. Effects of forskolin on intracellular cAMP concentration of porcine PE/NPE cells. 1-way ANOVA as unpaired data was used to compare the treatment groups with the control group, *$P < 0.05$, **$P < 0.01$.

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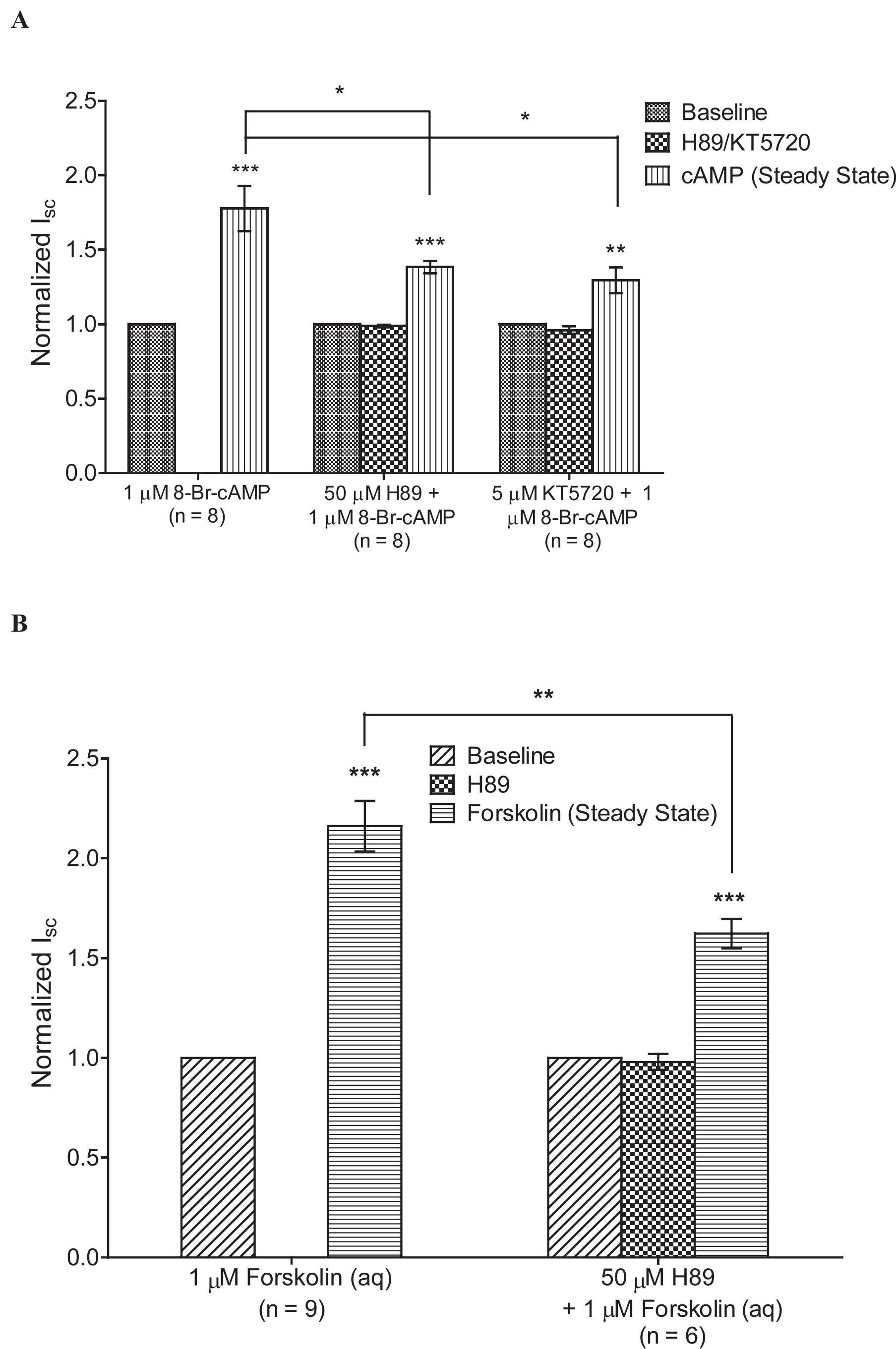

Figure 9. Inhibition of (**A**) cAMP- and (**B**) forskolin-stimulated I_{sc} by PKA blockers. PKA blockers (H89 or KT5720) were pretreated bilaterally for 30 minutes before the addition of cAMP or forskolin to the aqueous side. *$P < 0.05$, **$P < 0.01$.

Table 3. Inhibition of cAMP- and Forskolin-Stimulated I_{sc} by PKA Blockers

Condition	*n*	I_{sc}, μA·cm^{-2}		
		Baseline	Pretreatment of PKA Inhibitor H89/KT5720	8-Br-cAMP or Forskolin Administered With or Without PKA Inhibitor H89/KT5720
1 μM 8-Br-cAMP	8	10.6 ± 1.4	–	18.1 ± 2.1
50 μM H89 + 1 μM 8-Br-cAMP	8	10.4 ± 1.4	10.3 ± 1.4	14.2 ± 1.6
5 μM KT5720 + 1 μM 8-Br-cAMP	8	14.6 ± 1.5	14.0 ± 1.6	18.5 ± 1.9
1 μM forskolin	9	10.6 ± 1.9	–	23.7 ± 4.2
50 μM H89 + 1 μM forskolin	6	11.4 ± 1.0	11.2 ± 1.3	18.5 ± 1.3

Data are expressed as the mean ± SEM. *n* = number of experiments.

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transport mechanisms across the ciliary epithelium were reported.[17] For example, it has been demonstrated that the rabbit's ciliary epithelium primarily secretes HCO_3^- to drive aqueous humor formation, whereas in bovine and porcine preparations, Cl^- secretion seems to be the major driving force.[3,4,6,34] Therefore, it is important to compare results obtained from different mammalian species so that the full implications of these results for humans can be established.[17] It has been reported that cAMP and forskolin increased I_{sc} across the ciliary epithelia of rabbit,[35,36] monkey,[37] and pig.[16] However, an apparently opposing effect has been observed in bovine eyes, where cAMP inhibited the transepithelial secretion.[14] The secretion of aqueous humor is believed to be primarily driven by Cl^- transport in both bovine and porcine eyes, but the ionic dependency of bathing Cl^- and HCO_3^- concentrations, as well as the types of Cl^- channels present in the NPE cells, may be different between these two species.[17,34,38] In addition, the effect of cAMP on Cl^- transport varies with species. In the cow, cAMP has been shown to inhibit the net stromal-to-aqueous Cl^- flux,[14] whereas cAMP triggers a transient increase in net Cl^- flux across porcine ciliary epithelium.[16] Consistent with our current findings, it has recently been demonstrated that both cAMP and forskolin stimulate I_{sc} across the human ciliary body.[39] Likewise, the stimulatory effects are shown to be larger when cAMP/forskolin is added to the aqueous side versus the stromal surface.[39] Since human tissues are not easily accessible, these similarities support the notion that the pig could be a good animal model to mimic human aqueous humor secretion.[40–42]

In conclusion, cAMP induces an increase in I_{sc} and fluid transport across the porcine ciliary body and likely acts via the PKA signaling pathway. Nonpigmented ciliary epithelium cell Cl^- channels are a potential cellular target of cAMP-mediated responses. Understanding the fundamental aspects of aqueous humor secretion in the pig may provide us new insights for future development of glaucoma therapy.

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