Inhibitions of Chloride Transport and Gap Junction Reduce Fluid Flow across the Whole Porcine Ciliary Epithelium

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Purpose. To study the effects of chloride transport and gap junction inhibitors on fluid formation across the porcine ciliary epithelium.

Methods. A complete annulus of porcine iris–ciliary body preparation was mounted onto a modified Ussing type chamber to measure the fluid flow (FF) rate. The potential difference (PD) across the preparation was monitored simultaneously. The effects of several inhibitors on chloride transport and gap junction were studied. These included 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 5-(*N*,*N*-dimethyl)amiloride hydrochloride (DMA), bumetanide, niflumic acid, and heptanol.

Results. The average baseline FF rate was 2.56 ± 0.07 μL/h per preparation (n = 33). DIDS (0.1 mM) or DMA (0.1 mM) showed no effect on both FF and PD when added to the blood side of the preparation. Bumetanide (0.1 mM), on the blood side, inhibited the FF by 46% and caused a slight depolarization of PD. Heptanol (3.5 mM) depolarized the PD and reduced FF by 45% and 78% through the blood and aqueous sides, respectively. Niflumic acid (1 mM at the aqueous side) also depolarized the PD and significantly inhibited the FF (62%).

Conclusions. The effects of the chloride transport inhibitors on fluid formation across the porcine iris-ciliary body were comparable to that in previous chloride transport studies. The results indicated that fluid secretion by the isolated porcine ciliary epithelium is mainly driven by chloride transport. However, there may be other unidentified ion movements that drive residual FF after chloride transport is inhibited. (*Invest Ophthalmol Vis Sci.* 2009;50:1299–1306) DOI:10.1167/iovs.08-1888

The bilayered ciliary epithelium (CE) which secretes the aqueous humor (AH), consists of the pigmented epithelium (PE) facing the ciliary stroma and the nonpigmented epithelium (NPE) facing the posterior chamber. Most of the AH formation seems to be driven by active ion transport across the CE from the stromal to the aqueous side, and this resulting osmotic gradient generates a driving force for passive water flow. Therefore, the investigation of ion transport mechanisms of the CE has drawn significant attention in the study of AH formation.

Increasing evidence supports that active chloride (Cl^-) secretion contributes to the major driving force for AH formation.[1] Both the ox and the pig have a higher Cl^- concentration in the AH than in the blood plasma,[2] which indirectly indicates that the CE transports Cl^- from the blood into the AH. In addition, isotopic flux studies have demonstrated Cl^- secretion by the isolated bovine and porcine CE preparations,[3–5] which suggested that Cl^- transport is important for AH secretion. Moreover, the specific blockade of the transporters and channels in different CE surfaces inhibited Cl^- secretion across the CE preparations.[4,5] All these findings support the active Cl^- transport across the CE.

Our previous studies on the measurement of in vitro fluid flow (FF) through the isolated rabbit and bovine CE preparations have demonstrated that isolated CE preparations transport fluid in the blood-to-aqueous direction.[6] The FF is bicarbonate (HCO_3^-) dependent in the rabbit and Cl^- dependent in the bovine, as demonstrated with ion substitution on the blood-side bath. Later, we implemented a chamber system that simultaneously quantifies the volumetric FF and monitors the potential difference (PD) across the isolated porcine CE.[7] In addition to FF, PD measures the ionic equilibrium across the epithelium. Short-circuit current (I_{sc}), which reflects the active ionic transport activity of the epithelium, is directly proportional to PD. A depolarization of PD represents a decline of tissue resistance (R_t) or I_{sc}, which implies a reduction of transepithelial ion secretion. A decrease in I_{sc} can also occur when a cation is transported from the stroma to the aqueous side. This experimental setting serves as a model to elucidate the underlying relationship between the ionic transport activity, as reflected by PD, and the in vitro fluid movement, as reflected by FF. From our previous findings, the FF across the porcine CE is inhibited by ouabain, implying that the in vitro fluid movement is mainly driven by the active ion transport. Moreover, FF is dependent on both Cl^- and HCO_3^-, whereas the replacement of Cl^- has demonstrated a more drastic effect on FF, which is consistent with the Cl^--secreting properties of porcine CE.

In the present study, we conducted experiments to examine the effects of various Cl^- transport inhibitors on the FF and PD, to further characterize the Cl^- transporters in the porcine CE and to identify pharmacologic agents that could influence AH secretion in vivo. Several transport inhibitors have been shown to inhibit the FF and alter the PD across the CE, which supports the crucial role of Cl^- secretion in the formation of AH.

Materials and Methods

Isolation of the Iris-Ciliary Body

The dissection of iris-ciliary body was described in our previous report.[7] In brief, freshly enucleated porcine eyes were collected from a local abattoir and transported to the laboratory in ice. Extraocular tissues and muscles were completely removed from the globe. After

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the removal of cornea, the sclera was detached from the choroid by incision at the trabecular meshwork around the anterior angle and all the way to the equator. The intact ring of the iris-ciliary body with the choroid was separated from the posterior eyeball and placed in a Petri dish containing Ringer's solution. The vitreous humor remaining on the ciliary body was gently removed. The posterior lens capsule was incised, and the lens cortex was totally removed. The posterior lens capsule was trimmed away to the zonules, leaving behind the anterior lens capsule. The whole annulus of the iris-ciliary body was placed in Ringer's solution at room temperature.

Fluid Flow (FF) Chamber

The whole annulus of the porcine ciliary body was mounted in a modified Ussing chamber to measure FF across the preparation. The chamber was identical with the one we used in our previous work.[7] The chamber cavity was custom-made with the central post decentered, so as to accommodate the anatomic asymmetry of the porcine ciliary processes, which are wider at the temporal region and narrower at the nasal region. The isolated porcine ciliary body was transferred with a flat spatula to the circular hemichamber covered with tightly stretched nylon. The preparation was carefully aligned to the chamber cavity. The pupil (with lens capsule) was occluded with a circular Lucite block and the choroid was compressed between the paired hemichambers. Only the ciliary processes were exposed inside the chamber cavity (area of 1.28 cm^2) to the bathing solutions.

The hemichamber on one side had two ports on the top and bottom that connected to an external glass bubbler that enabled drug additions, changing of bath solutions and delivery of gas mixtures to circulate the bathing solution within the trough. A Ag/AgCl electrode (EKV; World Precision Instruments, Sarasota, FL) was also placed within the glass bubbler. On the other side, the upper port of the hemichamber was fitted with a connector that could hold a 25-μL capillary with graduations of 0.25 μL, so that changes in fluid volume inside the compartment could be visually detected. The capillary was pretreated with a hydrophobic agent (Rain-X; SOAPUS Products, Houston, TX) to prevent fluid crawl within it. A lower port on this hemichamber enabled connection to a second Ag/AgCl electrode, so that the pair of electrodes could be used to measure the PD across the preparation. The PD was continuously monitored by a dual-voltage clamp unit (DVC-1000; World Precision Instruments, Sarasota, FL) without clamping any current across the preparation. Experiments were conducted at a constant room temperature (temperature of the Ringer's solution during the experiments was stable at 23.5 to 24.5°C). It was previously found that at this temperature the FF was stable for at least 4 hours.

Measurement of FF Data

The capillary-containing hemichamber was a closed compartment, which was intended to foster an unstirred layer on the aqueous aspect of the tissue bathed within this compartment (as is the case in the posterior chamber of the eye) and enabled the detection of small changes in fluid volume inside the compartment. As such, solution changes and drug additions were made unilaterally on the side of the preparation containing the glass bubbler. The changes in capillary volume were recorded in 15-minute intervals and then converted to the rate of FF across the porcine CE.

As demonstrated in our last report,[7] the CE preparation transported fluid in the direction from blood to aqueous. This fluid movement reflected a consistent increase in the level of the capillary when it was placed on the aqueous side of the CE preparation, and a consistent decline in the capillary level when placed on the blood side. This FF was maintained for approximately 4 hours. Throughout the experiment, the capillary level was adjusted so that the difference between the capillary level and the water level of the glass bubbler was within 5 mm. When the capillary was placed on the aqueous side, the FF produced an increase in the capillary level, which then was manually lowered by removing the fluid. When the capillary was placed on the blood side, the decline in capillary level was compensated for by adding fluid to the capillary. This method ensured minimal pressure difference across the preparation.

In all experiments, 60 minutes was allowed for equilibration after mounting of the preparation. FF was measured for 90 minutes and was considered as the baseline recording. Then, a drug was added unilaterally to the glass bubbler to obtain the appropriate concentration, and 60 minutes was allowed for circulation and diffusion of the drug. FF was measured thereafter for another 60 minutes, and the FF measurement within the 60-minute period was averaged and considered as the drug-treatment recording. In separate experiments, the glass bubbler and capillary were interchanged so that the blood side or aqueous side of the preparation was facing the chamber containing the capillary. In all experiments, the side of drug addition was opposite that of the capillary.

Statistical Data Analysis

Data were expressed as the mean ± SEM. Paired Student's *t*-tests were used to analyze the FF measurement between the baseline and drug-treatment recordings to study the drug's effects.

Bathing Solutions

HEPES-buffered Ringer's solution was used for dissection and bathing the ciliary body preparation. It contained the following (in mM): 113.0 NaCl, 4.6 KCl, 21.0 NaHCO3, 0.6 $MgSO_4$, 7.5 D-glucose, 1.0 reduced glutathione, 1.0 Na_2HPO_4, 10.0 HEPES, and 1.4 $CaCl_2$. All chemicals used were of reagent grade. The solution was pre-equilibrated with 95% O_2 and 5% CO_2, and the pH was adjusted to 7.4.

Pharmacologic Agents

The pharmacologic agents used included 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 5-(*N*,*N*-dimethyl)amiloride hydrochloride (DMA), bumetanide, niflumic acid, and dimethyl sulfoxide (DMSO), all purchased from Sigma-Aldrich (St. Louis, MO) and heptanol, purchased from Fluka Chemie (Buchs, Switzerland). All chemicals were dissolved in DMSO before addition to the bathing solution in the glass bubbler, a single drug addition would add 0.1% of DMSO in the Ringer's solution, except heptanol, which was added directly into the bathing solution.

Results

We examined the effects of several transporter inhibitors: DIDS, DMA, bumetanide, heptanol, and niflumic acid on the FF rate and PD. The simultaneous FF and PD data were plotted as a function of time in individual figures. For each inhibitor, the representative experiment is shown in Figures 1 to 6.

The effects of different transporter inhibitors on the rate of FF across the isolated porcine CE preparation are summarized in Tables 1 to 6. The average baseline FF rate measured in the present study was 2.56 ± 0.07 μL/h per preparation (n = 33). It was similar to the control FF rate (2.72 ± 0.09 μL/h per preparation, n = 18) reported in our previous study,[7] and an unpaired *t*-test showed that there was no difference between the two measurements (P = 0.19).

Two major pathways have been proposed for the uptake of ions into the PE cell layer: Na^+/H^+ and Cl^-/HCO_3^- double exchangers and Na^+-K^+-$2Cl^-$ cotransporter (NKCC). To study the contribution of different ionic transport components in the fluid transport process in the CE, we tested three different agents: DIDS, DMA, and bumetanide added to the blood side of the preparation to study their effects on FF and PD. In the present study, additions of either DIDS (0.1 mM) or DMA (0.1 mM) to the blood side of the preparation showed no effect on both FF (Tables 1, 2) and PD (Figs. 1, 2). However, the addition of bumetanide (0.1 mM) on the blood side of the preparation

TABLE 1. Effect of Stromal Addition of 0.1 mM DIDS on FF across the CE Preparation

	FF Rate		
Experiment	**Baseline**	**Drug-Treatment**	**Change (%)**
1	2.33	2.25	−3.4
2	2.67	2.75	3.0
3	2.50	2.50	0.0
4	2.67	2.50	−6.4
5	2.00	2.25	12.5
Mean	2.43	2.45	0.8
SEM	0.12	0.09	

In each experiment, the FF rate (in μL/h per preparation) measured for 90 minutes before the addition of the drug was taken as the baseline. After the stromal addition of 0.1 mM DIDS, 60 minutes was allowed for stabilization, and the FF rate was measured thereafter for 60 minutes as the drug-treatment recording. $n = 5$.

inhibited the FF by 46% (Table 3) and caused a slight depolarization of PD (Fig. 3).

Since the coupling of PE and NPE layers by gap junctions is important in transepithelial ionic and water transport across the CE,[4,5,8,9] we applied heptanol to different sides of the preparation to study the effects of blockade of the gap junctions on both FF and PD. Heptanol (3.5 mM) reduced the FF by 45% (Table 4) and 78% (Table 5) when it was applied to the blood and aqueous sides, respectively. It also caused a significant depolarization of the PD when added to either side of the preparation (Figs. 4, 5). The action of heptanol was more dramatic when it was added on the aqueous side. It caused an inhibition of FF of 78% when it was applied to the aqueous side compared to 45% when added on the blood side. Moreover, the polarity of the PD was reversed subsequently during the aqueous addition of heptanol, implying a more drastic effect. The addition of heptanol induced an immediate depolarization of PD, whereas a mild delay in depolarization (approximately 15 minutes, as revealed in Fig. 4) was observed in the stromal addition.

In the subsequent step of transepithelial ionic transport, ions are released from the NPE cells into the AH through transporters or channels. Recently, it has been suggested that Cl^- efflux was associated with niflumic acid-sensitive Cl^- channels in porcine CE,[5] we therefore applied niflumic acid to study the effects of Cl^- channel blockade on both FF and PD in the same preparation. The addition of niflumic acid (1 mM) in the aqueous-side bath significantly inhibited the FF by 62% (Table 6) and caused a drastic depolarization of the PD (Fig. 6).

TABLE 2. Effect of Stromal Addition of 0.1 mM DMA on FF across the CE Preparation

	FF Rate		
Experiment	**Baseline**	**Drug-Treatment**	**Change (%)**
1	2.83	3.00	6.0
2	2.50	2.25	−10.0
3	2.83	2.50	−11.7
4	2.50	2.25	−10.0
5	2.00	2.25	12.5
Mean	2.53	2.45	−3.2
SEM	0.15	0.15	

In each experiment, the FF rate (in μL/h per preparation) was measured the same as described in Table 1, but with the stromal addition of 0.1 mM DMA. $n = 5$.

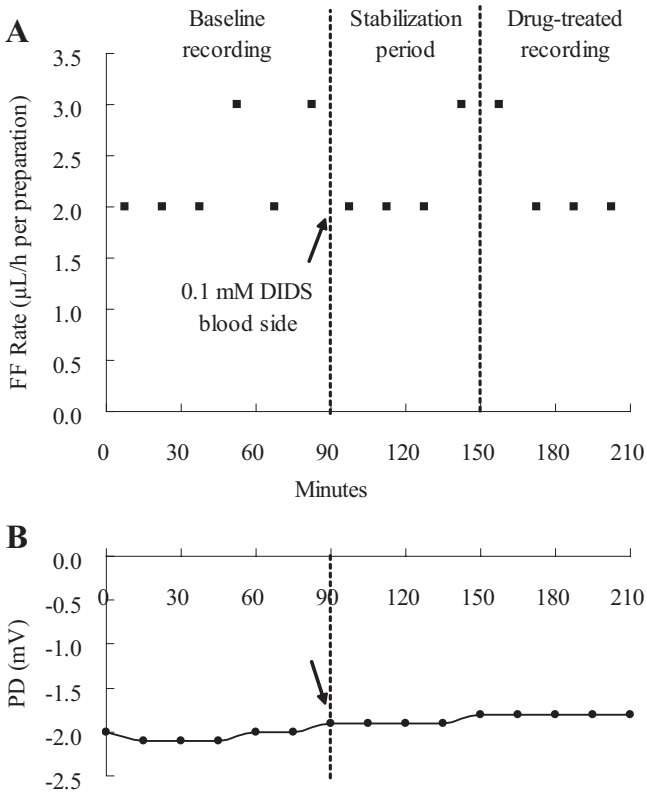

FIGURE 1. The representative experiment showing the effects of stromal addition of 0.1 mM DIDS on (**A**) FF and (**B**) PD, as a function of time. (**A**) The data points represent the calculated FF rate recorded in 15-minute intervals. The FF rate measured for 90 minutes before drug addition was taken as the baseline. After the stromal addition of DIDS, 60 minutes was allowed for stabilization and FF rate was measured thereafter for 60 minutes as the drug-treatment recording. (**B**) Data points represent the recorded PD from the preparation exposed to stromal DIDS. The polarity of the PD was consistently negative on the aqueous side relative to the blood side.

DISCUSSION

In the present and previous studies of FF, the whole-CE preparations from rabbit, ox, and pig transported only a small quantity of fluid in vitro. The isolated rabbit, bovine, and porcine CE preparations transported fluid at a rate ~3 μL/h per preparation.[6,7] Our current finding is also comparable to our previous report. The in vivo rate of AH formation in humans is approximately 165 μL/h,[10] which is ≈60-fold larger than the measured rate of the present study. This large discrepancy is observed in all studies with the in vitro preparations from rabbits, cows, and pigs. Several factors may be the reason for this discrepancy. There is no blood circulation in the isolated preparation, and the processes are collapsed. The chamber only clamps a fraction of the whole epithelium. Thus, the isolated tissue will not completely represent the in vivo system. In other studies in which the I_{sc} and net ion and water fluxes were measured, neither the respective I_{sc} or ion flux[4,5,11–13] is large enough to drive the aqueous flow observed in vivo, nor is the FF[6,7,14] of the magnitude measured in vivo. Except in the study conducted by Crook et al.,[15] the reported net Cl^- flux appears to be adequate to drive the AH secretion observed in vivo.

The key question of such observed discrepancy between electrical parameters and flux/FF is the relative contributions between active transport of CE and passive processes to AH formation. With the in vitro–perfused bovine eye model, ap-

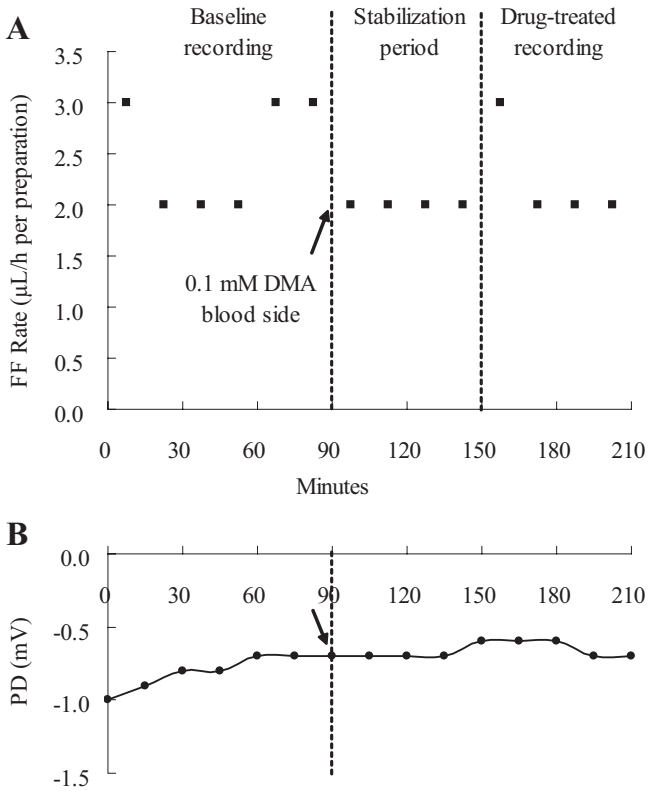

Figure 2. The representative experiment showing the effects of stromal addition of 0.1 mM DMA on (**A**) FF and (**B**) PD, as a function of time. (**A**) The data points represent the calculated FF rate recorded in 15-minute intervals. The FF rate measured for 90 minutes before drug addition was taken as the baseline. After the stromal addition of DMA, 60 minutes was allowed for stabilization and FF rate was measured thereafter for 60 minutes as the drug-treatment recording. (**B**) Data points represent the recorded PD from the preparation exposed to stromal DMA. The polarity of the PD was consistently negative on the aqueous side relative to the blood side.

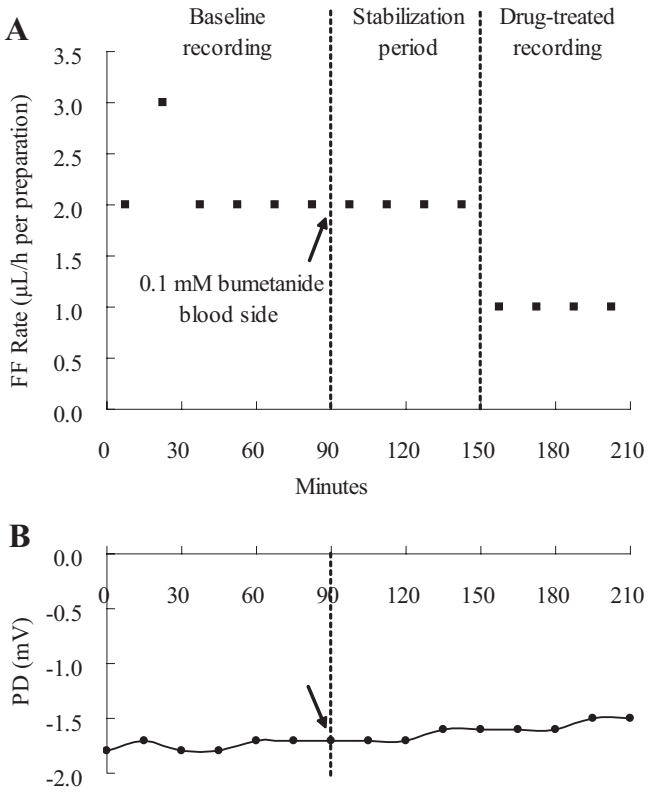

Figure 3. The representative experiment showing the effects of stromal addition of 0.1 mM bumetanide on (**A**) FF and (**B**) PD, as a function of time. (**A**) The data points represent the calculated FF rate recorded in 15-minute intervals. The FF rate measured for 90 minutes before drug addition was taken as the baseline. After the stromal addition of bumetanide, 60 minutes was allowed for stabilization and FF rate was measured thereafter for 60 minutes as the drug-treatment recording. (**B**) Data points represent the recorded PD from the preparation exposed to stromal bumetanide. The polarity of the PD was consistently negative on the aqueous side relative to the blood side.

proximately 60% of the AH secretion was mediated by the active ionic transport, which meant the remaining 40% was contributed by passive processes.[16] There is evidence suggesting that AH can be directly transferred into the anterior chamber via the anterior surface of the iris.[17,18] These findings suggested that AH may not be mainly secreted by an active transport process and the passive process may also play a significant role.

Table 3. Effect of Stromal Addition of 0.1 mM Bumetanide on FF across the CE Preparation

	FF Rate		
Experiment	**Baseline**	**Drug-Treatment**	**Change (%)**
1	2.33	1.00	−57.1
2	2.17	1.00	−53.9
3	2.83	1.50	−47.0
4	2.00	1.00	−50.0
5	3.17	2.25	−29.0
Mean	2.50	1.35*	−46.0
SEM	0.22	0.24	

In each experiment, the FF rate (in μL/h per preparation) was measured as described in Table 1, but with the stromal addition of 0.1 mM bumetanide. $n = 5$.

* Significantly lower than the baseline rate; $P < 0.001$, as paired data.

Although our in vitro FF of the ciliary body preparation represents only a small fraction of the in vivo AH formation rate, our present and previous experiments have shown that the CE preparation is viable.[4,19] The in vitro preparation demonstrated typical characteristics of a tight epithelium, with high transepithelial resistance (taking the area folding ratio into

Table 4. Effect of Stromal Addition of 3.5 mM Heptanol on FF across the CE Preparation

	FF Rate		
Experiment	**Baseline**	**Drug-Treatment**	**Change (%)**
1	3.00	2.00	−33.3
2	2.17	1.00	−53.9
3	2.17	1.50	−30.9
4	2.00	1.50	−25.0
5	3.50	1.25	−64.3
6	2.17	1.00	−53.9
Mean	2.50	1.38*	−44.8
SEM	0.25	0.15	

In each experiment, the FF rate (in μL/h per preparation) was measured as described in Table 1, but with the stromal addition of 3.5 mM heptanol. $n = 6$.

* Significantly lower than the baseline rate; $P < 0.001$, as paired data.

TABLE 5. Effect of Aqueous Addition of 3.5 mM Heptanol on FF across the CE Preparation

	FF Rate		
Experiment	**Baseline**	**Drug-Treatment**	**Change (%)**
1	2.67	1.00	−62.5
2	2.33	0.75	−67.8
3	2.17	0.50	−77.0
4	3.33	0.25	−92.5
5	2.17	0.50	−77.0
6	3.17	0.50	−84.2
Mean	2.64	0.58*	−78.0
SEM	0.21	0.11	

In each experiment, the FF rate (in μL/h per preparation) was measured as described in Table 1, but with the aqueous addition of 3.5 mM heptanol. $n = 6$.

* Significantly lower than the baseline rate; $P < 0.001$, as paired data.

consideration) as well as being able to conduct several active transport processes (ascorbate transport, sodium-pump activities, Cl^- transport and so on) known to occur in vivo. In our previous report,[7] the control FF was maintained stable for at least 4 hours. No further deterioration was observed within this period. In addition, the FF measured was abolished by stromal addition of ouabain (1 mM), which strongly suggested that the FF was primarily driven by an active ion transport process. Furthermore, the FF is inhibited by agents that act on the transporters and pathways known to be present in the epithelium, which may explain their possible effects on AH production and hence intraocular pressure in vivo.

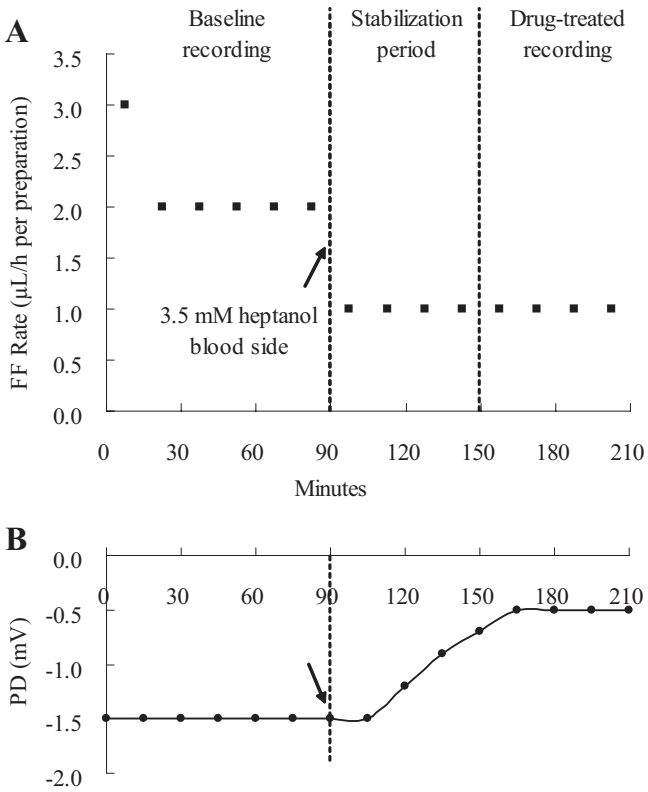

FIGURE 4. The representative experiment showing the effects of the stromal addition of 3.5 mM heptanol on (**A**) FF and (**B**) PD, as a function of time. (**A**) The data points represent the calculated FF rate recorded in 15-minute intervals. The FF rate measured for 90 minutes before drug addition was taken as the baseline. After the stromal addition of heptanol, 60 minutes was allowed for stabilization and FF rate was measured thereafter for 60 minutes as the drug-treatment recording. (**B**) Data points represent the recorded PD from the preparation exposed to stromal heptanol. The polarity of the PD was consistently negative on the aqueous side relative to the blood side.

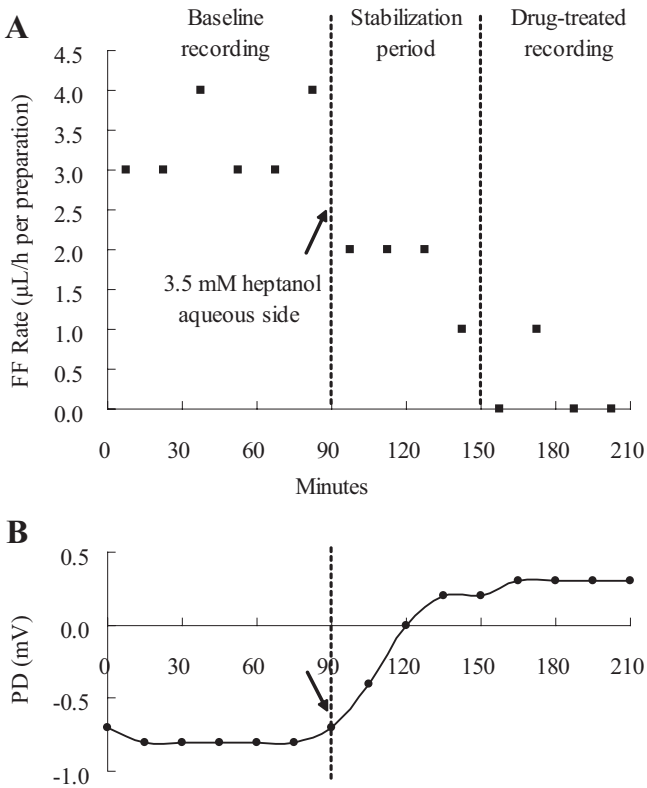

FIGURE 5. The representative experiment showing the effects of the aqueous addition of 3.5 mM heptanol on (**A**) FF and (**B**) PD, as a function of time. (**A**) The data points represent the calculated FF rate recorded in 15-minute intervals. The FF rate measured for 90 minutes before drug addition was taken as the baseline. After the aqueous addition of heptanol, 60 minutes was allowed for stabilization and FF rate was measured thereafter for 60 minutes as the drug-treatment recording. (**B**) Data points represent the recorded PD from the preparation exposed to aqueous heptanol. The polarity of the PD was consistently negative on the aqueous side relative to the blood side.

TABLE 6. Effect of Aqueous Addition of 1 mM Niflumic Acid on FF across the CE Preparation

	FF Rate		
Experiment	**Baseline**	**Drug-Treatment**	**Change (%)**
1	2.50	0.75	−70.0
2	3.00	1.50	−50.0
3	2.83	0.50	−82.3
4	2.33	1.25	−46.4
5	2.33	1.00	−57.1
6	3.17	1.25	−60.6
Mean	2.69	1.04*	−61.3
SEM	0.15	0.15	

In each experiment, the FF rate (in μL/h per preparation) was measured as described in Table 1, but with the aqueous addition of 1 mM niflumic acid. $n = 6$.

* Significantly lower than the baseline rate; $P < 0.001$, as paired data.

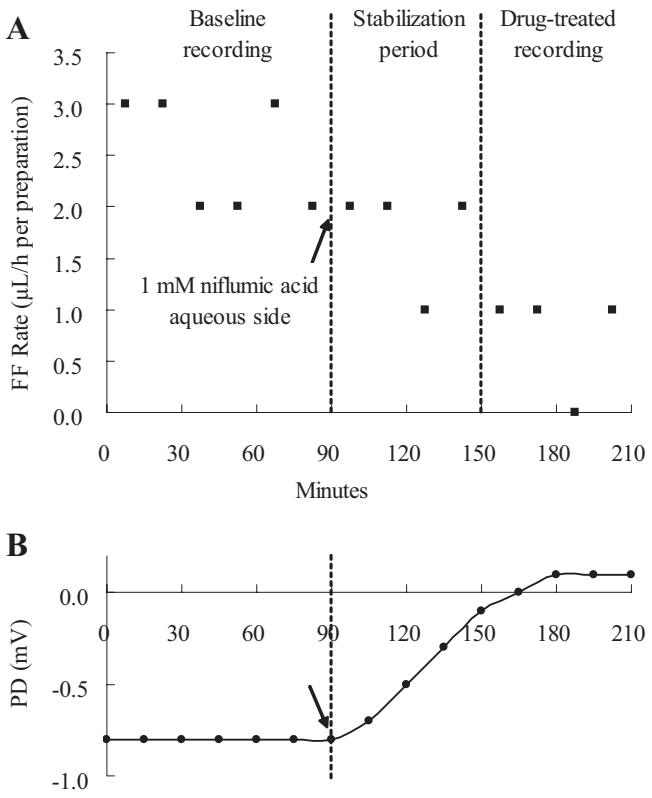

FIGURE 6. The representative experiment showing the effects of the aqueous addition of 1 mM niflumic acid on (**A**) FF and (**B**) PD, as a function of time. (**A**) The data points represent the calculated FF rate recorded in 15-minute intervals. The FF rate measured for 90 minutes before drug addition was taken as the baseline. After the aqueous addition of niflumic acid, 60 minutes was allowed for stabilization and FF rate was measured thereafter for 60 minutes as the drug-treatment recording. (**B**) Data points represent the recorded PD from the preparation exposed to aqueous niflumic acid. The polarity of the PD was consistently negative on the aqueous side relative to the blood side.

Uptake Pathway

There are two pathways proposed for the uptake of anions into the PE cells: Na^+/H^+ and Cl^-/HCO_3^- double exchangers and NKCC. The importance of the paired Na^+/H^+ and Cl^-/HCO_3^- exchanger system has been shown in rabbit CE, suggested that the paired exchangers play a role in the loading of HCO_3^- ions in the PE cells.[20] It is suggested that the double-exchanger system is predominant in rabbit CE, which plays a major role in the HCO_3^- transport across the epithelium.[20–23] Moreover, the Na^+/H^+ and Cl^-/HCO_3^- exchangers have been characterized in bovine PE cells.[24,25] The paired exchangers were suggested as the uptake pathway of both Na^+ and Cl^- ions in the bovine PE cells. However, recent electrophysiological studies show that the paired exchangers are not involved in transepithelial Cl^- secretion.[4,5] In the present study, the lack of effect of DIDS and DMA on both the FF and PD implies that the respective membrane transporters Cl^-/HCO_3^- exchanger and Na^+/H^+ exchanger are not functionally important in the transepithelial fluid transport process.

In the literature, little is known about these membrane transporters in the porcine CE, as the rabbit or ox was used as a model in most studies. Yet, we cannot exclude the presence of these transports in the epithelium, as they are known to be important in maintaining the normal cellular physiology such as regulating the intracellular pH.[26,27] We anticipate that the CE will have Cl^-/HCO_3^- exchanger and Na^+/H_+ exchanger for a housekeeping function. According to our data, the pair exchangers did not appear to contribute to fluid movement in vitro.

NKCC is predominantly localized along the PE cells in both the bovine and rabbit.[15,28] The functional importance of NKCC in bovine and porcine CE has been demonstrated in isotopic Cl^- flux studies.[4,5] Unexpectedly, Kishida et al.[29] and Crook et al.[15] have also found that the rabbit CE preparation secrets a net Cl^- flux which is different from the view that the rabbit CE transports HCO_3^- ions. In this study, the stromal addition of bumetanide, a specific blocker of NKCC significantly reduced the FF across the porcine CE. This observation implies that the NKCC system is the dominant system for Cl^- uptake and concomitant fluid secretion in the porcine CE preparation. However, the loop diuretics did not cause a drastic effect on PD. The observation that the PD did not decrease drastically with the FF is indeed very interesting. We have also observed this discrepancy in our previous Cl^- flux and transport experiments.[3–5] The main reason for this finding may be the electroneutral transport of NKCC. We have previously proposed that through detailed modeling with all the major transporters and channels, we may begin to correlate these parameters better.[30] The blockade of NKCC causes an unremarkable effect on both the PD and I_{sc} since both the uptake of cations and anions are reduced. However, the transepithelial Cl^- transport was eventually inhibited as a result of the reduced uptake of Cl^- ions, as shown in previous studies in both bovine and porcine CE.[3–5] In the present study, although bumetanide did not cause a drastic change in the PD across the CE, a marked reduction of FF was observed, which largely resembled the Cl^- transporting properties of porcine CE.

Gap Junction

Intercellular gap junctions are located between the PE and NPE cell layers, and between the adjacent cells within the PE and NPE layers.[31] This observation has lead to the view that the CE is a functional syncytium.[32] The functional role of gap junctions has been demonstrated in various studies.[4,5,8,9] In electrophysiological studies, addition of heptanol has been shown to inhibit I_{sc}, and inhibit Cl^- secretion by approximately 80% across the bovine and porcine CE.[4,5] In the present study, the application of heptanol to either side of the CE also depolarized the PD and inhibited the FF across the preparation. The maximum inhibition induced by aqueous addition of heptanol was 78%, which is in agreement with previous studies. Thus, the coupling of PE and NPE layers by gap junctions is essential in transepithelial ionic and fluid transport across the CE.

Since heptanol was able to inhibit the FF up to 78%, a major fraction of the FF measurement is attributable to the transcellular FF across the CE. This suggests that gap junctions are indispensable in the fluid transport process across the porcine CE. The actions of heptanol in reducing the FF is probably due to the interruption of intercellular communication between PE and NPE cells and hence the inhibition of the Cl^- secretion across the CE, thereby reducing the solute and osmotic gradient generated as the driving force for FF across the CE.

Furthermore, the functional importance of the gap junction is potentially linked to the regulatory action of the secondary messenger adenosine 3′,5′-cyclic monophosphate (cAMP) which has been shown to reduce Cl^- secretion in bovine CE.[33] However, further experiments are needed to determine the role of gap junction regulation in the formation of AH.

Cl^- Channels

Cl^- release from the NPE cells is the last step in transepithelial ion transport. The identity of Cl^- channels has been broadly investigated.[34,35] Recent studies of bovine CE cells has identified swelling-activated Cl^- channels in both the PE and NPE cells. cAMP-activated maxi-Cl^- channels are localized in the PE cells for recycling Cl^- and A_3 adenosine receptor-activated Cl^- channels in the NPE cells for releasing Cl^- into the AH.[35–37] In the present study, aqueous addition of niflumic acid inhibited FF by 62% and completely depolarized the PD. Compared to a previous study, niflumic acid completely abolished the I_{sc} and Cl^- secretion across the porcine CE.[5] Since the I_{sc} is directly proportional to the transepithelial PD, our results also indirectly showed that the I_{sc} was abolished by niflumic acid. However, if FF is totally dependent on Cl^- secretion, a complete abolishment of FF should be observed in association with a total inhibition of Cl^- secretion. That this abolition in FF did not occur in our experiments indicates that the fraction of FF not inhibited by niflumic acid (~38%) may be associated with other mechanisms of ionic transport.

Residual FF

Our findings indicate that in vitro FF of porcine CE is mediated largely by Cl^- secretion but none of the transport inhibitors was able to completely abolish the FF. The exact reason for the residual FF is unclear, which may represent fluid secretion that is driven by other ions. In addition, there are alternative channels or routes that are not inhibited by the above transport inhibitors by which Cl^- can go through.

Other than Cl^-, HCO_3^- is another candidate ion for the transport mechanism of fluid formation. In our previous study, an ion depletion experiment showed that FF across the porcine CE is HCO_3^- dependent to a certain extent.[7] Although our current findings have demonstrated that the Cl^-/ HCO_3^- exchanger is not active in the PE, there may be an unidentified transport mechanism of HCO_3^- present in the porcine CE that may drive FF. It is not surprising that a secretory epithelium transports various ions for secretion of fluid. For instance, the epithelial cells of the choroid plexuses transport several ions including Na^+, Cl^-, HCO_3^- from the blood to ventricles of the brain for the secretion of cerebrospinal fluid.[38] However, more study is needed to uncover the possible role of HCO_3^- in the porcine CE preparation.

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