

# Fluid Transport across the Isolated Porcine Ciliary Epithelium

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**PURPOSE.** To quantify spontaneous fluid transport across the isolated porcine ciliary epithelium and determine its sensitivity to the electrolyte transport inhibitors ouabain and bumetanide, as well as bath  $\text{Cl}^-$  and  $\text{HCO}_3^-$  levels.

**METHODS.** A complete annulus of ciliary body was mounted in a custom-designed chamber appropriate for quantifying net fluid movement, as well as the transepithelial potential difference (PD) across the in vitro ciliary epithelium.

**RESULTS.** A spontaneous and stable fluid flow (FF) in the blood-to-aqueous direction was measured over a 4-hour period. This flux solely reflected the secretory activity of the isolated ciliary epithelium (CE), given the absence of externally applied osmotic or pressure gradients. In contrast to FF, the PD declined during the 4 hours in vitro, suggesting that the integrity of the tight junctions may have been compromised during this time so that an increased movement of counter ions via the paracellular pathway could have shunted the PD, while at the same time transcellular fluid transport remained unaffected. The FF in the blood-to-aqueous direction ( $2.3 \pm 0.2 \mu\text{L/hr}$ ;  $n = 7$ ) was eliminated by a unilateral reduction in the bath  $\text{Cl}^-$  levels on the blood side of the preparation and restored on reintroducing the anion to the bathing medium. This linkage between FF and blood side  $[\text{Cl}^-]$  is consistent with the existence of a net  $\text{Cl}^-$  flux across the porcine CE in the same direction as the fluid transport. Addition of bumetanide to the blood-side bath inhibited FF by  $\approx 40\%$ , whereas the removal of  $\text{CO}_2/\text{HCO}_3^-$  from the blood-side bathing solution elicited a  $\approx 50\%$  reduction in FF. Ouabain inhibited the FF from either side of the preparation, although the effects were more rapid when the glycoside was applied to the blood side of the tissue. Overall, these findings indicate the dependence of FF on active ionic transport by the isolated CE.

**CONCLUSIONS.** Isolated porcine ciliary epithelial preparations transport fluid in the blood-to-aqueous direction, indicating that measurements of volumetric fluid flow across this preparation may serve as a suitable model for future studies directed toward the pharmacological control of secretion. (*Invest Ophthalmol Vis Sci.* 2007;48:321-327) DOI:10.1167/iovs.06-0432

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The driving forces for fluid production across the ciliary epithelium are the osmotic gradient (created by ionic transport mechanisms) and a possible hydrostatic pressure difference. The relative contribution of these forces to the production of aqueous humor is still an open question, although most researchers accept a contribution from both.<sup>1-4</sup> Only three studies have measured the actual spontaneous movement of fluid, which occurs secondary to active transport mechanisms, across the isolated ciliary epithelium.<sup>5-7</sup> All quantified a fluid transport rate that was many times less than the generally accepted rate of in vivo aqueous formation. The large discrepancy between the in vivo and in vitro rates of fluid production does not necessarily indicate that ultrafiltration is the major component for aqueous humor formation, as discussed in detail earlier.<sup>7</sup> In short, it may reasonably be supposed that the transport activity of the isolated ciliary epithelium is unlikely to be identical with that in the intact animal. Nevertheless, in vitro measurements of fluid transport are potentially useful as they offer the opportunity to characterize the secretory activity of the epithelium in the absence of osmotic or pressure gradients.

Our previous studies on this topic measured a net fluid flow in the blood-to-aqueous direction across isolated rabbit and bovine ciliary epithelia.<sup>7</sup> It was observed that tissue freshness is a critical parameter for detection of this flow. The spontaneous, baseline fluid transport rate could last as long as 4 hours, but it generally declined after 2 hours, as also found by others.<sup>6</sup> After corrections for area and possible collapse of the processes, a total flux rate of approximately  $23 \mu\text{L/h}$  or 13% of the in vivo flow in rabbit was estimated.<sup>7</sup> This value agrees with predictions from ionic fluxes and short-circuit current measurements, which are also obtained in vitro.<sup>8-10</sup> The fluid flow is bicarbonate dependent in the rabbit and chloride dependent in the bovine, consistent with ionic transport mechanisms described in these respective species.<sup>7</sup>

In the present study, we repeated this characterization in porcine ciliary epithelium, which is also a  $\text{Cl}^-$ -secreting tissue (Kong MCW, et al. *IOVS* 2003;44:ARVO E-Abstract 3428). Overall, we found this preparation more durable than those of the rabbit or cow. The porcine preparation maintained a relatively steady fluid transport rate in vitro over a 4-hour period. Because the  $\text{Cl}^-$  levels of the porcine aqueous are higher than those of plasma,<sup>11</sup> as also found in humans,<sup>12-14</sup> and this finding is consistent with a net  $\text{Cl}^-$  transport across the ciliary epithelium in the blood-to-aqueous direction (Kong MCW, et al. *IOVS* 2003;44:ARVO E-Abstract 3428), the isolated pig ciliary epithelium appears to be a more suitable model (than rabbit or bovine) for future studies directed toward the pharmacological control of the secretory activity of the epithelium.

## MATERIALS AND METHODS

Freshly enucleated porcine eyes were collected from a local abattoir in Hong Kong and transported to the laboratory of one of the authors (C-HT) at the Hong Kong Polytechnic University on ice. All extraocular tissues and muscles were removed, leaving behind the globe. The cornea was removed by cutting circumferentially  $\approx 2$  mm anterior to

the limbus. The remnant of the cornea at the limbal region was gripped carefully with a forceps, exposing the anterior angle of the eye. The sclera was detached from the iris base by incision at the trabecular meshwork around the anterior angle and was further separated by cutting along the choroid-scleral interface all the way to the equator of the globe. The eye was then cut in half anteroposteriorly, leaving behind an intact ring of ciliary body with choroid that was placed, with ciliary process facing upward, in a Petri dish containing Ringer's solution. Remaining vitreous humor on the ciliary body was removed gently. The posterior lens capsule was incised, and the lens cortex was removed. The posterior lens capsule was removed by trimming all the way to the zonules, leaving the remaining anterior lens capsule.

The complete annulus of the iris-ciliary body (I-CB) preparation was transferred with a flat spatula and placed on a circular Lucite hemichamber covered with tightly stretched nylon. The preparation was carefully aligned to the chamber cavity. Its pupil (with lens capsule) was occluded by a circular Lucite block before placing a paired hemichamber over the preparation to hold it in place.

The structures of the Lucite hemichambers were described and illustrated previously.<sup>7</sup> We used similar chambers with minor modifications. The cavity within one hemichamber comprised an outer circular trough (16.0 mm in diameter) and an inner central post (12.5 mm in diameter). Both the central post and the circular trough were notched to hold an O-ring in the same plane. A small circular Lucite block, with a diameter matching the central post, was machined to hold an O-ring and four pins. The block was screwed into the central post for securing the iris and enclosing the pupil. On placement of the paired hemichamber, the outer circular troughs within each hemichamber were aligned so that only the ciliary processes (area of 1.28 cm<sup>2</sup>) were exposed to the chamber cavity and bathing solutions. Because of the anatomical asymmetry of the ciliary processes, which are wider at the temporal region and narrower at the nasal region, the central post was decentered to the outer trough wall to accommodate this asymmetry.

Ports were drilled into the side walls of the chambers to connect the fluid in the troughs to external vessels. The hemichamber on one side had two ports that led 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. An Ag/AgCl electrode (EKV; World Precision Instruments, Sarasota, FL) was also placed within the glass bubbler. On the other side, an upper port on the hemichamber was fitted with a connector that could hold a 25- $\mu$ L capillary with graduations of 0.25  $\mu$ L, 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 the capillary. A lower port on this hemichamber enabled connection to second Ag/AgCl electrode, so that the pair of electrodes could be used to measure the potential difference (PD) across the preparation. The PD was continuously monitored by a dual-voltage clamp unit (DVC-1000; World Precision Instruments).

The lack of a provision for gas circulation within the enclosed capillary-containing hemichamber was a design intended to foster an unstirred layer on the epithelial aspect of the tissue bathed within this compartment, as well as to enable the detection of small changes in chamber volume. As such, solution changes and drug additions were made unilaterally on the side of the preparation containing the glass bubbler.

After mounting the I-CB preparation, a bathing solution that had been pre-equilibrated with 5% CO<sub>2</sub> and 95% O<sub>2</sub> was pipetted into the respective side hemichambers through either the glass bubbler (8 mL total volume) or through the port and connector (0.9 mL total volume). After filling, the graduated capillary tube was inserted into the connector for measurement. Gas (5% CO<sub>2</sub> and 95% O<sub>2</sub>) was delivered through the glass bubbler continuously. The I-CB preparation was maintained at room temperature (temperature of Ringer's solution was 23.5–24.5°C). Room temperature was chosen as an experimental condition to obviate any possible change in the capillary levels due to temperature

imbalances between the bathing solutions that may occur on heating the circulating bubbled-side bath and the stagnant capillary-side medium.

After waiting 30 minutes for equilibration, the capillary level was recorded at 15-minute intervals and the rate of fluid flow (FF) was calculated. In separate experiments, the complete annulus of I-CB was oriented between the hemichambers so either the blood side or the ciliary process-side interfaced with the chamber containing the capillary.

## Bathing Solutions

HEPES-buffered Ringer's solution (NR) was used for dissection and bathing the I-CB preparation. It contained the following (in mM): 113.0 NaCl, 4.5 KCl, 21.0 NaHCO<sub>3</sub>, 0.6 MgSO<sub>4</sub>, 7.5 D-glucose, 1.0 reduced glutathione, 1.0 Na<sub>2</sub>HPO<sub>4</sub>, 10.0 HEPES, and 1.4 CaCl<sub>2</sub>. The solution was pre-equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and the pH was adjusted to 7.4. For chloride (Cl<sup>-</sup>) substitution experiments, the Cl<sup>-</sup> concentration in the Ringer's solution was reduced from 120 mM (in the NR) to 7 mM by replacing the NaCl with an equimolar amount of Na-glucuronate. The latter compound was also used as a substitute for NaHCO<sub>3</sub> in some experiments requiring bicarbonate-free conditions with 100% O<sub>2</sub> bubbling.

## Pharmacological Agents

Ouabain and bumetanide (purchased from Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl sulfoxide and ethanol, respectively, each at  $\geq$ 100-fold solutions, before addition to the bathing solutions.

## Statistical Analysis

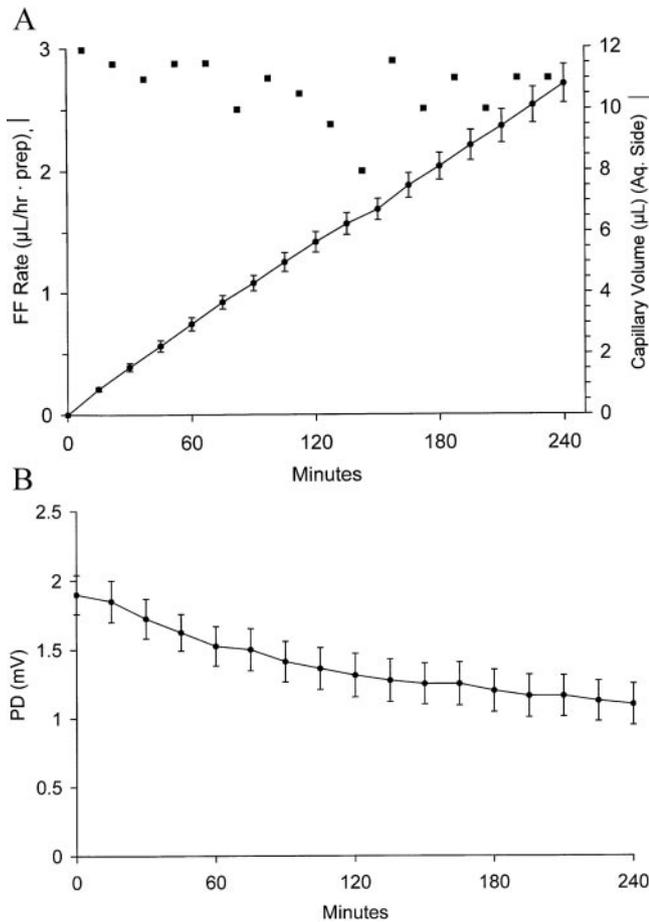
Data are expressed as the mean  $\pm$  SEM and statistically analyzed with Student's *t*-test, either as paired or unpaired data, or ANOVA, as noted.

## RESULTS

We have built a modified Ussing-type chamber to measure simultaneously the FF and PD across the isolated pig ciliary epithelium (CE). Although the intact I-CB is mounted within the chamber, the iris is pressed against the Lucite walls holding the tissue in place, so that there is minimal or no contact between the iris and the bathing solutions. Under these conditions, the compartmental volume of the blood side gradually decreased, while that of the aqueous side simultaneously increased. This represents a fluid secretion in the blood-to-aqueous direction across the CE.

The average changes in the capillary volume of the aqueous (Fig. 1A,  $n = 8$ ) and blood (Fig. 2A,  $n = 10$ ) sides were recorded as a function of time. The calculated FF rates (expressed as microliters per hour per preparation) of the respective time intervals were also plotted (solid squares). The FF rate declined slightly over time. The volumetric increase of the aqueous-side compartment was  $\sim$ 3.4  $\mu$ L/h per preparation in the first 15 minutes and declined to 2.8  $\mu$ L/h per preparation after 4 hours (linear equation from regression analysis:  $y = -0.0017x + 2.9061$ ). Similarly, the volumetric decrease from the blood-side compartment was initially 3.60  $\mu$ L/h per preparation and declined to 2.50  $\mu$ L/h per preparation after 4 hours (linear equation:  $y = -0.0027x + 2.945$ ).

For statistical analysis, the FF data measured over 4 hours were divided into two major time frames (i.e., a first and second period), with each representing approximately 2 hours. The initial 15-minute value was excluded because of its large variation, which may have reflected a variable equilibration period within the chamber. The remaining FF rates for each major time frame were averaged, and the differences between the two were compared. When the capillary tube was connected to the aqueous-side compartment ( $n = 8$ ), the mean FF was  $2.75 \pm 0.16$   $\mu$ L/h per preparation during the first



**FIGURE 1.** Spontaneous fluid movement across the isolated porcine ciliary epithelium in the blood-to-aqueous direction and the simultaneously recorded transepithelial PD. (A) *Left axis:* the calculated fluid flow (FF) rate, expressed per hour during the course of the experiment. *Right axis:* the measured change in the capillary level on the aqueous side of the preparation plotted as a function of time. Data points are the mean  $\pm$  SE of results of eight control experiments. (B) The value of the PD recorded every 15 minutes simultaneously with the measurement of the capillary levels (A). Points are the mean  $\pm$  SE ( $n = 8$ ). The polarity of the PD was consistently negative on the aqueous side relative to the blood side.

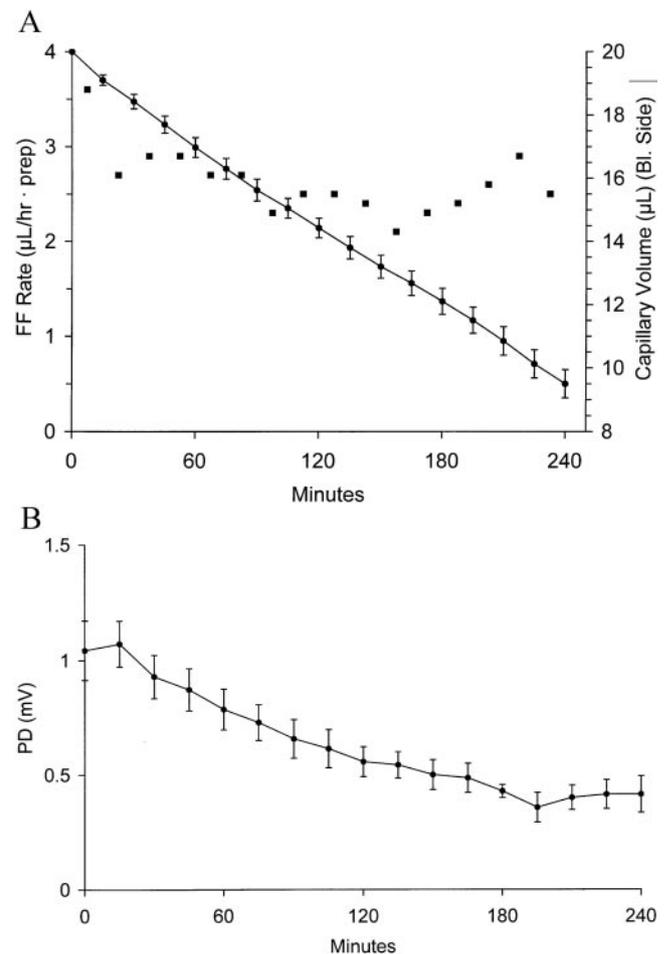
period and  $2.64 \pm 0.19 \mu\text{L/h}$  per preparation during the second (means  $\pm$  SE;  $P > 0.4$ , as paired data), suggesting that the preparation was stable for at least 4 hours. With the capillary tube placed in the blood-side compartment ( $n = 10$ ), the mean FF was  $2.69 \pm 0.11$  and  $2.46 \pm 0.10 \mu\text{L/h}$  per preparation for the first and second periods, respectively ( $P > 0.1$ , as paired data). There was no difference in the measured FF rates ( $P = 0.45$ , one-way ANOVA, as unpaired data) with the capillary on either the aqueous or blood sides of the preparations.

The standing PDs (in mV) across the CE preparations during these experiments with the capillary on the aqueous (Fig. 1B) and blood (Fig. 2B) sides are plotted as a function of time. The polarity of the PD was consistently negative on the aqueous side. It declined gradually but slowly with time. From the experiments with the capillary on the aqueous side, the PD was initially 1.94 mV and declined to 1.09 mV after 4 hours, whereas with the capillary on the blood side, the decrease was from 1.04 to 0.41 mV.

It was noteworthy that the FF rate was rather constant with time during 4 hours in vitro, but the PD declined. This suggests that the integrity of the tight junctions may have been compromised during this time in vitro so that an increased move-

ment of counter ions via the paracellular pathway could have shunted the PD, while at the same time transcellular fluid transport remained unaffected.

$\text{Cl}^-$  substitution experiments were conducted to evaluate the role of  $\text{Cl}^-$  in the process of fluid transport across the porcine CE. The effect of reduced  $[\text{Cl}^-]$  in the blood-side bath on blood-to-aqueous fluid transport is shown in Table 1. With the halide concentration reduced, the FF rate declined from  $2.26 \pm 0.18$  to  $-0.57 \pm 0.20 \mu\text{L/h}$  per preparation ( $n = 7$ ;  $P < 0.001$ , as paired data), indicating, on average, a reversal in flow, so that a net movement occurred in the aqueous-to-blood direction. Restoring the  $[\text{Cl}^-]$  by reintroducing NR to the blood-side hemichamber reversed the FF to  $1.70 \pm 0.08 \mu\text{L/h}$  per preparation, a rate  $\approx 25\%$  lower than baseline ( $P < 0.05$ , as paired data). Among the seven individual experiments, this  $\text{Cl}^-$  reduction protocol abolished the FF in two preparations, whereas with the remaining five, the direction of the FF reversed (Table 1).



**FIGURE 2.** Spontaneous fluid movement across the isolated porcine ciliary epithelium measured with the capillary on the blood side of the preparation, along with the simultaneously recorded transepithelial PD. (A) *Left axis:* the calculated FF rate in the blood-to-aqueous direction, expressed per hour during the course of the experiment. *Right axis:* the measured change in the capillary level on the blood side of the preparation plotted as a function of time. Note that the capillary level declined, in contrast to the orientation used with the experiments shown in Figure 1. Data points are mean  $\pm$  SE of results in 10 control experiments. (B) PD recorded every 15 minutes simultaneously with the measurement of the capillary levels (A). Data are the mean  $\pm$  SE ( $n = 10$ ). The polarity of the PD was consistently negative on the aqueous side relative to the blood side.

**TABLE 1.** Effects of Reduced  $\text{Cl}^-$  Concentration of the Stromal Bath on Fluid Flow across the Porcine Ciliary Epithelium in the Blood-to-Aqueous Direction

Exp.	Baseline (NR)	Low $[\text{Cl}^-]$ (7 mM $\text{Cl}^-$ )	Recovery (NR)
Cl-1	2.37	0.00	1.60
Cl-2	3.00	-0.20	1.80
Cl-3	2.46	-1.20	1.30
Cl-4	1.83	-0.30	1.60
Cl-5	2.14	-1.30	1.80
Cl-6	2.00	-1.00	2.00
Cl-7	2.00	0.00	1.80
Mean	2.26	-0.57*	1.70*
SEM	0.18	0.20	0.08
n	7	7	7

Fluid flow (FF) is expressed as  $\mu\text{L}/\text{hr}$  per preparation and represents the spontaneous volumetric movement of fluid across the epithelium in the blood-to-aqueous direction. A negative value for FF indicates a net fluid flow in the reverse, aqueous-to-blood direction.

All tissues were pre-equilibrated and bathed bilaterally in the divided chamber with  $\text{HCO}_3^-$ -buffered NR solution plus 5%  $\text{CO}_2$  bubbling of the blood-side hemichamber opposite to the aqueous-side compartment containing the capillary. The FF rate measured during an initial period of 75 minutes was taken as the baseline. Thereafter, the solution on the stromal side of the preparation was replaced with the low  $[\text{Cl}^-]$  medium and a stabilization period of 30 minutes was allowed, before the measuring of FF for an additional 60 minutes. For the recovery conditions, FF was measured 30 minutes after the reintroduction of NR over a period of an additional hour.

\* Significantly different than respective antecedent,  $P < 0.001$ , as paired data.

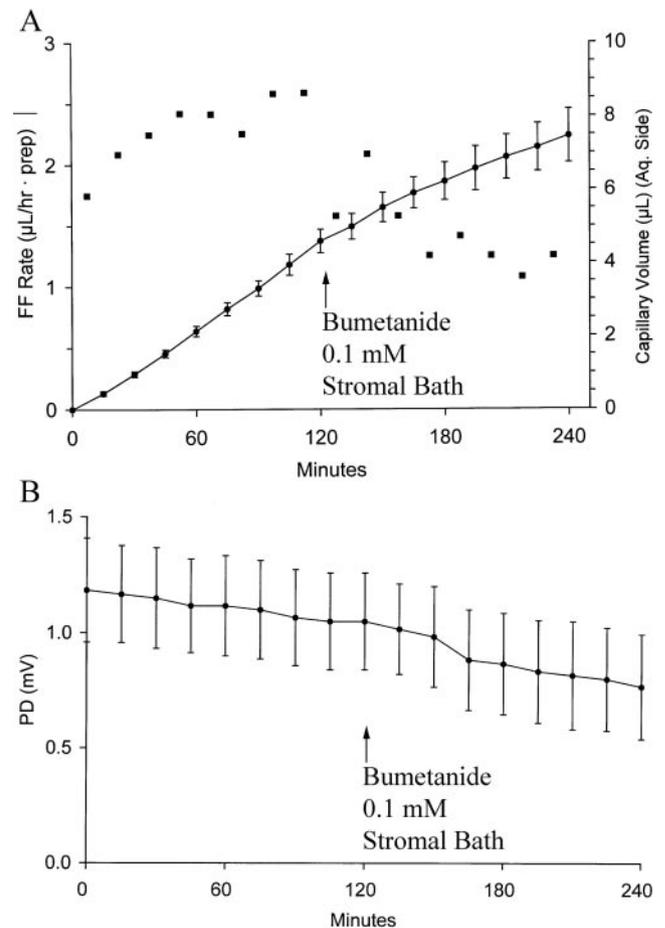
The marked dependency of FF on blood-side  $\text{Cl}^-$  levels (Table 1) is consistent with the concept that transport of the anion across the preparation in the aqueous direction underpins the fluid flow. To assess this likelihood, the effects of bumetanide, a highly selective inhibitor of the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  cotransporter (NKCC, which appears to mediate a significant fraction of the net  $\text{Cl}^-$  transport across the bovine preparation<sup>15</sup>) were tested on the porcine FF and PD.

Addition of 0.1 mM bumetanide to the blood-side bath reduced FF by 40% (from  $2.37 \pm 0.08$  to  $1.42 \pm 0.14 \mu\text{L}/\text{h}$  per preparation;  $n = 6$ ;  $P < 0.001$ , as paired data). Figure 3A shows a plot of the changes in the capillary levels that were recorded during these experiments and the reduction in slope ( $\Delta$  capillary volume/ $\Delta$  time) on addition of the loop diuretic to the stromal bath. The calculated FF rates, which are plotted as solid squares between each recorded time interval (Fig. 3A), were averaged after excluding the FF values for the intervals, 0 to 15 minutes and 120 to 135 minutes, to obtain the means reported herein. It was clear that a steady rate of FF was maintained in the presence of bumetanide for the overall interval 180 to 240 minutes, suggesting that either NKCC was not completely inhibited in the porcine preparation by the dose of bumetanide applied or that other mechanisms such as the parallel activities of the  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  exchangers could contribute to FF.<sup>15</sup>

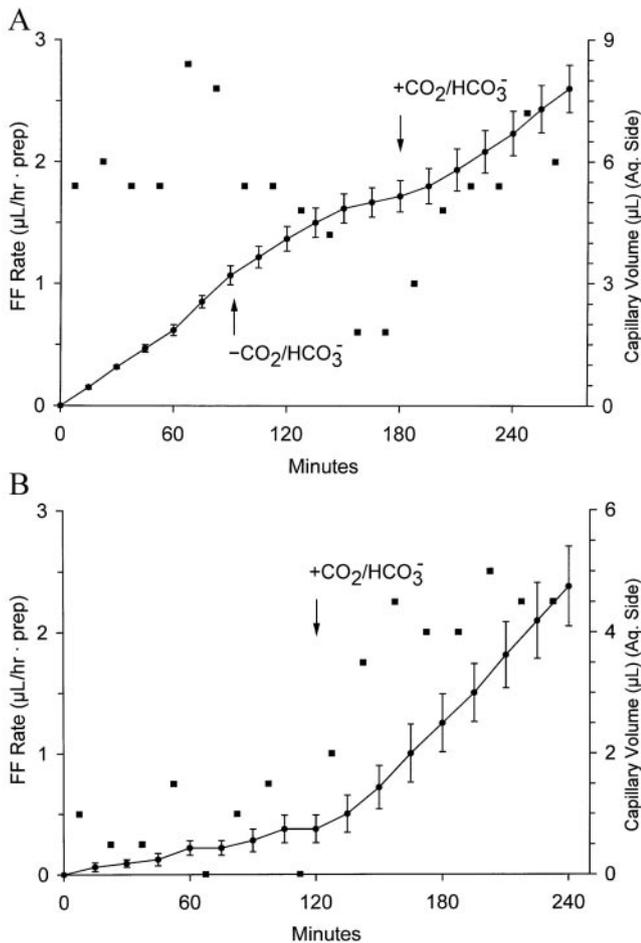
As mentioned earlier (Figs. 1B, 2B), the PD during control intervals gradually declined. In the case of the bumetanide set of experiments (Fig. 3B), the observed PD decline was not as extensive, given that the value at  $t = 120$  minutes ( $1.05 \pm 0.21$  mV;  $n = 6$ ), was not significantly lower than the value at  $t = 0$ ,  $1.18 \pm 0.22$  mV;  $P > 0.16$ , as paired data). Although the PD at 240 minutes,  $0.77 \pm 0.23$  mV, is indeed lower than that at  $t = 120$  ( $P < 0.001$ , as paired data), a dramatic change in PD on the addition of bumetanide at  $t = 120$  minutes was not observed.

In addition to a blood-side  $[\text{Cl}^-]$  requirement for fluid transport (Table 1), the in vitro FF across the porcine preparation was also linked to the presence of  $\text{CO}_2/\text{HCO}_3^-$  in the stromal bath (Fig. 4A). Removal and reintroduction of  $\text{CO}_2/\text{HCO}_3^-$  from the blood-side hemichamber (by unilaterally washing out the NR with  $\text{HCO}_3^-$ -free solution at  $t = 90$  minutes plus switching to 100%  $\text{O}_2$  gas bubbling, followed by restoration of the control conditions at  $t = 180$  minutes) elicited a reversible, 52% reduction in FF. In these experiments (Fig. 4A), the baseline FF was calculated for 90 minutes, thereby obtaining a mean of six 15-minute intervals from five preparations of  $2.17 \pm 0.17 \mu\text{L}/\text{h}$  per preparation. Under  $\text{CO}_2/\text{HCO}_3^-$ -free conditions, FF declined significantly to  $1.04 \pm 0.11 \mu\text{L}/\text{h}$  per preparation (mean excludes the first interval after the solution change;  $P < 0.002$ ), and recovered to  $2.02 \pm 0.10 \mu\text{L}/\text{h}$  per preparation on reintroducing the control bathing conditions on the stromal side of the preparation.

Given indications for a linkage between blood-side bicarbonate levels and FF, a second set of experiments was conducted whereby the tissues were bathed bilaterally with the HEPES-buffered,  $\text{HCO}_3^-$ -free medium with 100%  $\text{O}_2$  bubbling of the blood-side bath and the capillary on the aqueous-side



**FIGURE 3.** Effects of bumetanide on the spontaneous fluid movement across the isolated porcine ciliary epithelium in the blood-to-aqueous direction and transepithelial PD. (A) *Left axis:* the calculated flow rate. *Right axis:* the measured change in the capillary level on the aqueous side of the preparation. Data are the mean  $\pm$  SE of results in six preparations. Bumetanide was introduced unilaterally after the recording of the capillary level at  $t = 120$  minutes. (B) Data points are the mean  $\pm$  SE of the simultaneously recorded PD from the six preparations exposed to bumetanide. The polarity of the PD was consistently negative on the aqueous side relative to the blood side.



**FIGURE 4.** Effects of  $\text{CO}_2/\text{HCO}_3^-$  on the spontaneous fluid movement across the isolated porcine ciliary epithelium in the blood-to-aqueous direction. (A) *Left axis:* the calculated flow rate plotted as squares. *Right axis:* the measured change in the capillary level on the aqueous side of the preparation. Data are the mean  $\pm$  SE of results from five preparations. *Arrows:* the points at which  $\text{CO}_2/\text{HCO}_3^-$  were removed and reintroduced to the blood-side bath. (B) Data from a set of four experiments in which the ciliary epithelia were bathed bilaterally with HEPES-buffered,  $\text{HCO}_3^-$ -free medium plus 100%  $\text{O}_2$  bubbling of the blood-side bath and the capillary on the aqueous-side hemichamber.  $\text{CO}_2/\text{HCO}_3^-$ -rich medium was used to replace the baseline, blood-side solution at the point indicated (*arrow*).

hemichamber (Fig. 4B). Under these conditions, fluid transport in the blood-to-aqueous direction was  $0.39 \pm 0.11 \mu\text{L/h}$  per preparation ( $n = 4$ ), a relatively low rate, suggesting an important role for bicarbonate in maintaining FF under baseline conditions. The flow immediately increased on replacement of the blood-side bathing medium (aqueous side left unaltered) with NR containing 21 mM  $\text{HCO}_3^-$  plus 5%  $\text{CO}_2$  gas bubbling to  $2.21 \pm 0.18 \mu\text{L/h}$  per preparation ( $P < 0.001$ , as paired data;  $n = 4$ ); an FF rate comparable to that obtained with tissue pre-equilibrated and maintained under  $\text{CO}_2/\text{HCO}_3^-$ -rich conditions (e.g., Figs. 1A, 2A).

The PD was not notably affected by the unilateral removal and/or addition of bicarbonate (not shown). This is not unexpected, given that in open-circuit conditions, changes in the levels in one bath of a charged species that can traverse both transcellular and paracellular pathways, could produce ambiguous and irrelevant PD responses.

The effects of ouabain, a specific inhibitor of  $\text{Na}^+/\text{K}^+$ -ATPase activity, were also determined on the FF rate and PD across the porcine CE (Table 2). Its addition at 1 mM to the blood side

( $n = 6$ ) elicited a 97% inhibition in FF ( $P < 0.001$ , as paired data) and a biphasic PD response (i.e., a slight depolarization that was promptly followed by a more substantial hyperpolarization lasting for 1 hour (Fig. 5A); thereafter, the PD gradually depolarized. The addition of 1 mM ouabain to the aqueous side ( $n = 6$ ) also caused transient hyperpolarization in PD (Fig. 5B), along with a 54% reduction in FF ( $P < 0.001$ , as paired data; Table 2). Thus, the PD effects of ouabain were largely similar when applied to either the aqueous or blood sides. However, the effects on FF rates differed significantly. Although an exact explanation of these diverse effects requires further study, it was clear that the driving force for FF was not eliminated by aqueous-side ouabain within the 2-hour period that the capillary levels were monitored in the presence of the glycoside (plots not shown).

## DISCUSSION

With a volumetric technique similar to one that we developed earlier for measurements of spontaneous fluid flows across isolated rabbit and bovine ciliary body preparations,<sup>7</sup> the present study represents the first report of a spontaneous fluid transport by the porcine ciliary epithelium and its influence by bath  $\text{Cl}^-$  and  $\text{HCO}_3^-$  levels. This fluid movement solely reflects the secretory activity of the isolated ciliary epithelium because the in vitro arrangement precludes contributions from ultrafiltration, as well as externally applied osmotic or pressure gradients. The linkage between FF and the  $[\text{Cl}^-]$  of the stromal bath is consistent with a net  $\text{Cl}^-$  flux in the aqueous direction that was measured across porcine CE preparations isolated in divided chambers comparable to those used presently (Kong MCW, et al. *IOVS* 2003;44:ARVO E-Abstract 3428) and congruent with the inhibitory effects of bumetanide on FF (Fig. 3A). As such, the spontaneous, transmural volume change detected under control conditions appears to represent, at least in part, a net fluid transport secondary to active  $\text{Cl}^-$  transport by the porcine ciliary epithelium.

In addition, the porcine CE may also mediate a net  $\text{HCO}_3^-$  transport in the same direction as that of  $\text{Cl}^-$ . However, corroborative evidence for this idea is currently lacking, and the present experiments are not sufficient to substantiate it. Solution changes were made unilaterally, because the chamber design necessary for monitoring FF required a closed capillary-

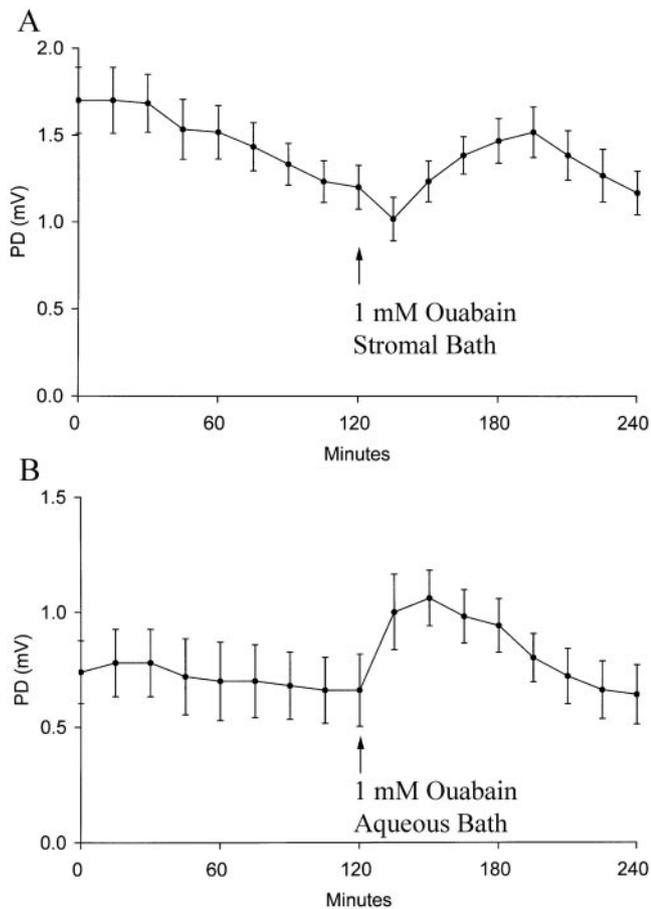
**TABLE 2.** Sidedness of Effects of 1 mM Ouabain on Fluid Flow across the Porcine Ciliary Epithelium

	n	FF Rate		
		Baseline	Treated	Inhibition
Blood side	6	$2.39 \pm 0.21$	$0.07 \pm 0.22^*$	97%
Aqueous side	5	$2.37 \pm 0.06$	$1.09 \pm 0.11^*$	54%

FF rate is expressed as microliters per hour per preparation and represents flow in the blood-to-aqueous direction. With the capillary on the aqueous side (allowing for addition of ouabain to the blood side of the tissue), data indicate that the capillary level increased; conversely, with the capillary on the blood side (allowing for ouabain addition to the aqueous side of the tissue), the capillary decreased.

The FF rate measured over an initial 2-hour period before the addition of the drug was taken as the baseline. Following the unilateral ouabain introduction, 30 minutes were allowed to elapse prior to the retaking of FF measurements for an additional 90 minutes. The simultaneously recorded changes in PD that were obtained during these experiments are shown in Fig. 5. Note that the PD was not eliminated by 2-hours of exposure to the glycoside.

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



**FIGURE 5.** Biphasic responses of the PD across porcine ciliary epithelium upon the addition of ouabain to either the blood side (A) or ciliary processes-side (B) of the preparations. The polarity of PD was negative on the aqueous side relative to the blood side. Arrows: the time points of ouabain addition. The simultaneously recorded effects of ouabain on fluid transport during these sets of experiments are given in Table 2.

side compartment. As such, a significant movement of  $\text{HCO}_3^-$  across both trans- and paracellular pathways from the  $\text{HCO}_3^-$ -rich bath to the opposite-side solution may have contributed to (1) the reduction in FF obtained on removing  $\text{CO}_2/\text{HCO}_3^-$  from the blood-side hemichamber (Fig. 4A), and (2) the increase in FF on introducing  $\text{CO}_2/\text{HCO}_3^-$  to the blood-side bath of tissues pre-equilibrated under bicarbonate-free conditions (Fig. 4B). Nevertheless, the partial inhibitory effect of bumetanide (Fig. 3A) implies the presence of an additional ionic transport mechanism to buttress the fluid flow. Either a net bicarbonate transport indeed occurs, or  $\text{Cl}^-$  fluxes in the aqueous direction could be maintained secondary to the generation of cellular  $\text{H}^+$  and  $\text{HCO}_3^-$  (from metabolic  $\text{CO}_2$  production and endogenous carbonic anhydrase activity), which are then exchanged with  $\text{Na}^+$  and  $\text{Cl}^-$  via parallel  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  antiporters in the PE, as suggested in the case of the bovine CE.<sup>16</sup> This possible mechanism would have been precluded during the initial baseline period with bicarbonate-free solutions and 100%  $\text{O}_2$  gassing in the experiments shown in Figure 4B, thereby resulting in the markedly lower rate of fluid transport exhibited by preparations under these conditions.

Changes in bath  $\text{Cl}^-$  levels were also unilateral (Table 1), but as noted earlier, evidence for net  $\text{Cl}^-$  transport by the porcine CE in vitro has been obtained (Kong MCW, et al. *IOVS* 2003;44:ARVO E-Abstract 3428). Further studies such as those

that we have done earlier with the bovine CE,<sup>16</sup> in which we measured unidirectional  $\text{CO}_2/\text{HCO}_3^-$  fluxes across the preparation and determined the effects of  $\text{CO}_2/\text{HCO}_3^-$  on unidirectional  $^{36}\text{Cl}^-$  fluxes are needed to elucidate the underlying transport mechanisms in the porcine tissue.

In approximately one third of the isolated porcine preparations, FF was not detectable. It was generally observed that tissue freshness was a critical factor favoring fluid transport. This consideration, plus the chamber design, which limited prompt changes in bath composition to only one side of the preparation, indicates to us that our approach is best suited for relatively straightforward tests aimed at determining whether a given pharmaceutical affects FF directly. Agents of interest (e.g.,  $\beta$ -blockers) could be added in separate experiments, to either the stromal or aqueous sides of the preparations—the latter determined with the central annulus oriented in a manner so that the capillary-containing hemichamber interfaces with the stromal side of the preparation, as in Figure 2A.

Yet, it is also important to note that fluid transport by isolated CE preparations<sup>5-7</sup> represents only a small fraction of the rate of aqueous humor formation. Although we are unaware of measurements for the rate of aqueous humor formation in the pig, the reported rates of aqueous humor production by humans and rabbits are  $\approx 165$  and  $180 \mu\text{L}/\text{h}$ , respectively, or  $\approx 60$ -fold larger than that measured in the present study. Because not all the processes and pars plana are included in the surface exposed to the bathing medium, it is difficult to compare accurately the in vitro and in vivo rates.

Moreover, the large discrepancy between in vitro and in vivo fluid transport also suggests the importance of additional factors. One obvious consideration is that hydrostatic pressure may be an important driving force in vivo by inducing ultrafiltration, as previously proposed.<sup>1-5</sup> Alternatively, it is also possible that in the isolated preparation, without blood supply, the rate of active transport decreases. This may be due to a partial collapse of the ciliary processes in the divided chambers, and/or the possibility that the blood circulation provides hormones or other paracrine agents that stimulate active ionic transport, which are obviously not present in the in vitro bathing solutions. These putative shortcomings were also inherent under the conditions of earlier attempts to quantify spontaneous fluid transport across the isolated rabbit ciliary body.<sup>5-7</sup>

In addition, it may be that not all aqueous humor production arises from fluid movement across the ciliary processes. The fact that proteins directly enter the anterior chamber by crossing the anterior surface of the iris<sup>17</sup> implies that fluid may also passively enter via this route.

At the same time, however, the present study did not demonstrate a large role for ultrafiltration in the in vivo production of aqueous humor; some other factors, as discussed, may be involved, so that ultrafiltration does not necessarily represent the large difference between the in vivo and in vitro fluid transport values. Nor does it appear likely that the discrepancy is attributable to the fact that our experiments were conducted at room temperature. At lower temperature, the nutritional supply to the CE may have been more adequate to maintain ionic transport than in previous in vitro studies performed at physiological body temperatures.

The transepithelial electrical parameters of control preparations declined, but subsisted over 3 to 4 hours, suggesting that the driving force for spontaneous fluid transport was not lost within this period in vitro. The polarity of the PD was negative on the aqueous side relative to the blood side of the porcine CE, as reported earlier with isolated I-CB preparations of cat,<sup>18</sup> rabbit,<sup>8,19</sup> and bovine.<sup>15</sup> This parameter indicates a net movement of anions across the CE to the aqueous-side bath. With the exception of the rabbit species,<sup>7</sup>  $\text{Cl}^-$  is most

likely the predominant anion transported, as a net  $\text{Cl}^-$  secretion has been demonstrated in the bovine<sup>15</sup> and porcine CE (Kong MCW, et al. *IOVS* 2003;44:ARVO E-Abstract 3428), and there are presently no direct indications of a net transport of other anions or of a net movement of cations in the opposite direction. Consistent with a net flux of  $\text{Cl}^-$  across the porcine CE in the blood-to-aqueous direction, steady state  $\text{Cl}^-$  levels of the porcine aqueous humor are higher than plasma.<sup>11</sup>

The overall effects of ouabain on the PD were largely similar when applied to either the blood- or aqueous-side baths, but its inhibitory effects on FF were more pronounced on its addition to the bathing medium of the former side. Although further characterization is needed to explain this phenomenon, the following notion seems plausible. It has been demonstrated that the two cell layers exhibit the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  enzyme,<sup>20-23</sup> and, in other studies, ouabain elicits a prompt depolarization of the trans-PD across the CE on its addition to the blood side and a hyperpolarization when added to the aqueous bath.<sup>6,8</sup> These initial changes commonly reverse as the glycoside diffuses across the preparation and inhibits the pump activity on the opposite-side cell layer. The initial depolarization obtained on adding ouabain to the blood-side bath (Fig. 5A) was not as pronounced as reported by others,<sup>6,8</sup> as it was rapidly followed by a hyperpolarization, because either diffusion of ouabain across the porcine preparation is faster than across other preparations, or the levels of ouabain used in the present study (1 mM) enhanced the delivery of the inhibitor to the opposite-side cell layer. The attenuation of the  $\text{Na}^+$  gradient within the bilayered CE may have occurred faster when ouabain was added to the blood-side bath, so that the inhibitory effects on FF were more evident (Table 2; in contrast, there was only a 54% reduction in FF after 2 hours of aqueous-side ouabain exposure). The sidedness of the effectiveness of ouabain is reminiscent of the greater potency of bumetanide when applied from the blood side of the bovine CE.<sup>15</sup>

The main finding of the present study is that the porcine CE preparation exhibited more sustained rates of FF than those obtained with CE preparations of bovine and rabbit.<sup>7</sup> Although the absolute values for FF among the preparations of the three species were largely similar, FF across the bovine and rabbit preparations gradually declined within 3 to 4 hours.<sup>6,7</sup> This loss in fluid transport function by the bovine and rabbit preparation in vitro was accelerated by the presence of ouabain. In contrast, the porcine preparations were relatively robust in that FF could be maintained in vitro for 4 hours under control conditions.

Overall, the present observations indicate that the measurements of volumetric fluid flow across the porcine ciliary epithelium are suitable for future studies directed toward the pharmacological control of the secretory activity of the epithelium. It would be important to determine the effects of pharmacological agents on fluid transport to confirm their postulated effect in vivo. Hypothetically, drugs known to reduce aqueous humor formation as determined by fluorophotometry, may or may not affect fluid transport. A demonstration of a similar effect on in vitro FF would establish that their action is mediated by inhibition of ionic transport mechanisms. A lack of an effect on in vitro fluid transport would be an indication that in vivo there are additional important components involved in the production of aqueous humor.

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