J. Membrane Biol. 182, 213–222 (2001)
DOI: 10.1007/s00232-001-0045-x

The Journal of
Membrane
Biology

Regional Differences in Ciliary Epithelial Cell Transport Properties

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Received: 4 December 2000/Revised: 24 April 2001

Abstract. Experiments were performed to determine whether the transport properties of the ciliary epithelium vary over different regions. Rabbit iris-ciliary bodies were incubated under experimental or control conditions for 30 min before quick freezing, cryosectioning, dehydration and electron probe X-ray microanalysis. Cryosections were cut from three regions along the major axis of the iris-ciliary body, i.e., the anterior, middle and posterior (pars plicata) regions. In bicarbonate/CO_2 solution, the epithelial cells of the anterior and middle regions contained more Cl and K than did those of the posterior region. These higher levels of Cl and K were reduced by the carbonic anhydrase inhibitor acetazolamide. Application of bumetanide, an inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter, resulted in significant increases in Cl and K in the anterior and middle regions but not in the posterior region. In bicarbonate-free solution, the ratio for K/Na contents was higher in the posterior than in the two more anterior regions; Na, K and Cl contents of epithelial cells in the three regions were otherwise similar. Cell composition did not differ significantly between the crests and valleys of the posterior region. The divergent responses to perturbation of epithelial transport in the different regions provide the first demonstration of functional heterogeneity along the major axis of the iris-ciliary body. The response to inhibition of carbonic anhydrase raises the possibility that the anterior aspect of the ciliary epithelium may be the major site of aqueous humor secretion.

Key words: Electron probe X-ray microanalysis — Na^+-K^+-$2Cl^-$ symport — Cl^-/HCO_3^- antiport — Na^+/H^+ antiport — Carbonic anhydrase — Chloride content

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Introduction

The eye's aqueous humor is formed by the bilayered ciliary epithelium (Krupin & Civan, 1995), as partially illustrated in Fig. 1*A*. The first step in secretion is electroneutral transfer of NaCl from the stroma of the ciliary processes to the pigmented ciliary epithelial (PE) cells, which can be mediated by either paired NHE-1 Na^+/H^+ and AE2 Cl^-/HCO_3^- exchangers (Wiederholt, Helbig & Korbmacher, 1991; Kaufman & Mittag, 1994; McLaughlin et al., 1998; Counillon et al., 2000) or a Na^+-K^+-$2Cl^-$ co-transporter (Wiederholt & Zadunaisky, 1986; Edelman, Sachs & Adorante, 1994; Do & To, 2000; Crook et al., 2000; Dunn, Lytle & Crook, 2001). Consensus has not yet been reached concerning the relative importance of these two mechanisms. Gap junctions connect the cells of the bilayer, so that it functions as a syncytium (Bowler et al., 1996; Carré et al., 1992; Coca-Prados et al., 1992; Edelman et al., 1994; Green et al., 1985; Mitchell & Civan, 1997; Oh et al., 1994; Raviola & Raviola, 1978; Walker et al., 1999; Wiederholt & Zadunaisky, 1986; Wolosin & Schütte, 1998). Following passage of Na^+ and Cl^- into the nonpigmented ciliary epithelial (NPE) cells, Na^+ is extruded through Na^+,K^+-activated ATPase and Cl^- is released through Cl^- channels into the aqueous humor. NaCl may be secreted through an additional electroneutral mechanism (Chu & Candia, 1987), which may involve the Na^+-K^+-$2Cl^-$ symporter (McLaughlin et al., 1998). Inhibition of Na^+ release through the Na^+,K^+-activated ATPase, using either cardiotonic steroids or application of metabolic inhibitors, markedly inhibits secretion (Krupin & Civan, 1995).

With rare exception (Civan, Coca-Prados & Peterson-Yantorno, 1996; Civan et al., 1997; Sears, 1984), net secretion is taken to equal unidirectional secretion of aqueous humor. Nevertheless, mechanisms which could support transfer of solution in the opposite direction,

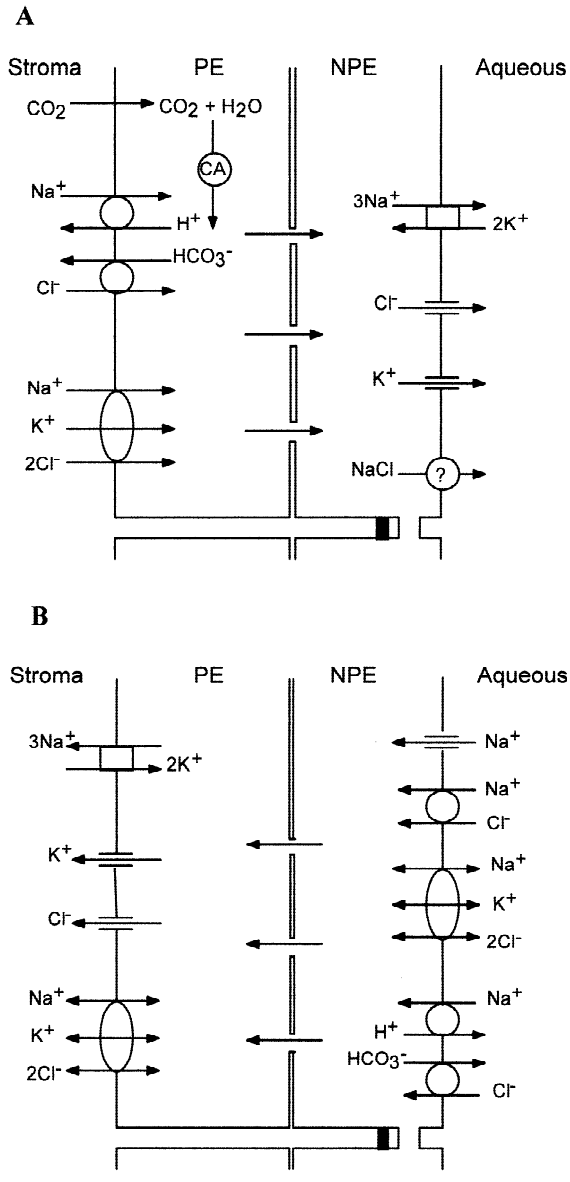

Fig. 1. (*A*) Consensus model of NaCl secretion by the rabbit ciliary epithelium, possibly operational in the anterior regions. Adapted from Counillon et al. (2000). Paired Na^+/H^+ and Cl^-/HCO_3^- antiporters, or the $Na^+K^+2Cl^-$ symporter, can support uptake from the stroma into the PE cells. Gap junctions provide entry to the NPE cells, and release into the aqueous humor occurs via the Na^+,K^+-exchange pump, Cl^- channels and an electroneutral mechanism. (*B*) The locations of ciliary epithelial cell membrane transporters reported by others, possibly conferring a reabsorptive function in the posterior region; Na^+/H^+ and Cl^-/HCO_3^- antiporters, Na^+-K^+-$2Cl^-$ and Na^+-Cl^- symporters and amiloride-sensitive Na^+-channels may provide entry to the NPE cells, while Na^+,K^+-ATPase and Cl^- channels located at the PE cells may support release into the stroma.

from aqueous humor to stroma (reabsorption), have been observed (Fig. 1*B*). NPE cells in culture display functional evidence of Na^+/H^+ and Cl^-/HCO_3^- antiports, Na^+-K^+-$2Cl^-$ and Na^+-Cl^- symports, and amiloride-sensitive Na^+-channels (Crook, von Brauchitsch & Polansky, 1992; Von Brauchitsch & Crook, 1993; Crook & Polansky, 1994; Dong & Delamere, 1994; Crook & Riese, 1996; Civan et al., 1996; Riese et al., 1998). Furthermore, PE cells of the intact epithelium also express Na^+,K^+-activated ATPase (Coca-Prados & Sanchez-Torres, 1998) and cultured PE cells have Cl^- channels which can be synergistically activated by ATP and tamoxifen (Mitchell et al., 2000). Thus, mechanisms are in place for reabsorption of aqueous fluid, and net formation of aqueous humor could represent a balance between unidirectional secretion and reabsorption.

This balance between unidirectional transfer into and out of the aqueous humor may vary within the different regions of the ciliary epithelium. A number of reports have noted regional differences in the expression of Na^+/K^+-ATPase and other proteins and biologically active peptides (Dunn et al., 2001; Eichhorn, Flügel & Lütjen-Drecoll, 1990; Eichhorn & Lütjen-Drecoll, 1993; Flügel et al., 1993; Flügel & Lütjen-Drecoll, 1988; Flügel et al., 1989; Ghosh et al., 1990, 1991). For example, the NPE cells of young calves display higher expression of $\alpha_1/\alpha_2/\alpha_3/\beta_1/\beta_2$ isoforms of Na^+,K^+-activated ATPase in the anterior than in the posterior region of the ciliary epithelium (Coca-Prados & Sánchez-Torres, 1998). In contrast, the PE cells display a uniform relative concentration of α_1/β_1 throughout the intact epithelium, prompting a suggestion that in the posterior region, net ion transport may occur towards the stroma (Ghosh et al., 1991). In addition, the Na^+-K^+-$2Cl^-$ cotransporter was localized to the basolateral edge of the PE cell layer in the anterior region of young calves, with reduced evidence of the cotransporter either posteriorly or on the NPE cells (Dunn et al., 2001). Until now, it has not been possible to test whether these regional differences in structure are associated with differences in function because of the structural complexity of the iris-ciliary body.

We have addressed the issue with electron-probe X-ray microanalysis (Dörge et al., 1978; Hall, 1971; Rick, Dörge & Thurau, 1982) of the intact rabbit iris-ciliary body. This approach, which measures the intracellular elemental content within identifiable cellular sites of the NPE and PE cells (Bowler et al., 1996; McLaughlin et al., 1998) also identifies the topographical origin of the cells so analyzed and is one of very few techniques capable of addressing the typography of cellular function. The results, which identify some transport pathways as playing a major role in the anterior region, but with diminished activity posteriorly, provide the first functional evidence of topological heterogeneity of the ciliary epithelium.

Materials and Methods

The methods used in this study have been described in detail elsewhere (Bowler et al., 1996; McLaughlin et al., 1998).

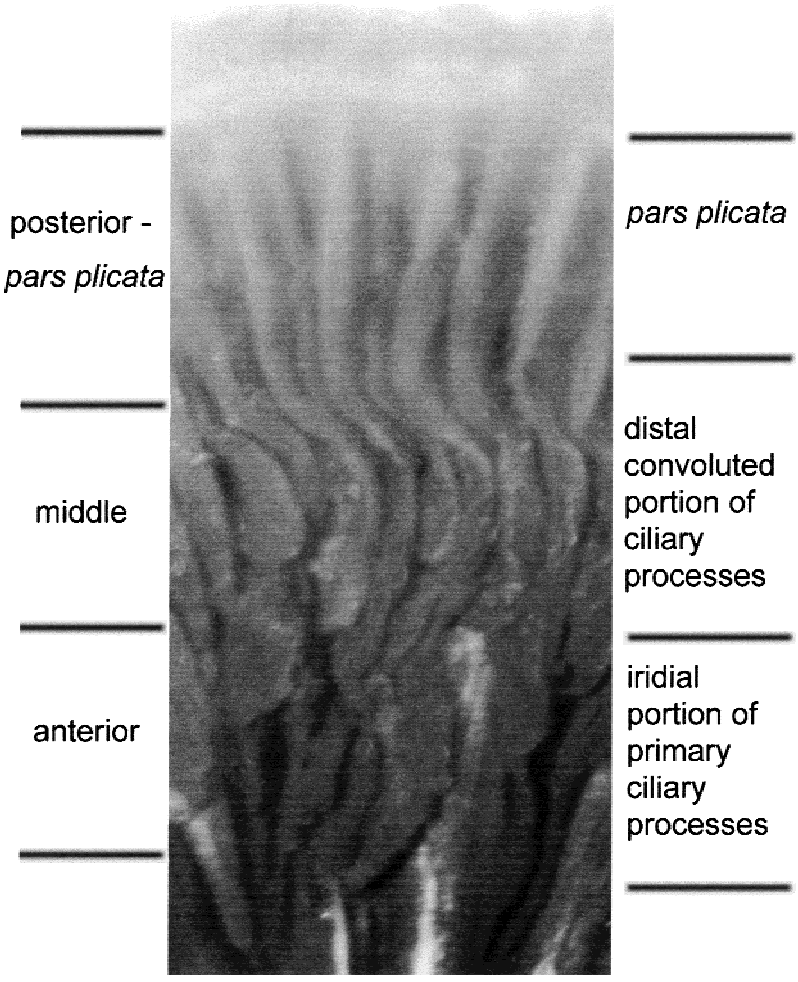

Fig. 2. Macrophotograph of a portion of the rabbit iris-ciliary body showing the gross morphology of the structure and illustrating the relationship between the areas analyzed and the terminology of Weingeist (1970).

Cellular Model

Dutch-belted rabbits of either sex and older than 6 weeks post-weaning were obtained from the Department of Laboratory Animal Sciences, University of Otago Medical School, and were treated in accordance with the ARVO Resolution on the Use of Animals in Research. Animals were anesthetized with 30 mg/kg sodium pentobarbital and sacrificed by injecting air into the marginal ear vein. After enucleation, the iris-ciliary body was excised, cut into quarters and each quarter bonded at its edge to a plastic frame with cyanoacrylate. Pairs of quadrants (one from each eye) were incubated in beakers for $\geqslant$ 2 hr in bicarbonate or bicarbonate-free solution as appropriate, then for at least 30 min under the different experimental conditions. Incubation was conducted at room temperature (18–22°C) as discussed elsewhere (McLaughlin et al., 1998).

After incubation, the tissues were blotted then plunged into liquid propane at −180°C to freeze the preparation quickly before ions and water could undergo any redistribution. Blocks were fractured from the frozen tissue under a dissecting microscope (×7), while paying careful attention to the origin and orientation of the block. After transfer of the block to the cryoultramicrotome and consequent trimming, we were thus able both to select and identify accurately the region from which the sections were being cut. The rabbit iris-ciliary body displays some structural differences from ciliary bodies of bovine or human origin, but remains a preferred experimental model. Its structure has been well documented by a number of workers (Kozart, 1968; Lütjen-Drecoll & Lonnerholm, 1981; Prince, 1964; Sheppard, 1962; Wegner, 1967; Weingeist, 1970) who have proposed various nomenclatures. We find some of these (Kozart, 1968; Lütjen-Drecoll & Lonnerholm, 1981; Weingeist, 1970) particularly relevant to our own observations, and use the terms suggested by Weingeist (1970). In our experiments reported to date, we had chosen to analyze epithelial cells in sections obtained from what has been termed (Weingeist, 1970) the ciliary processes (Figure 2). However, we subsequently noted that different cellular compositions may be measured in sections that had occasionally been cut from more posterior regions of the iris-ciliary body. To examine the possibility of heterogeneity in the distribution of transport pathways in the epithelium, we have now systematically analyzed sections obtained from three parts of the iris-ciliary body (Fig. 2); the posterior *pars plicata* (Weingeist, 1970) region adjacent to the minimal (in the rabbit) *pars plana* (Weingeist, 1970), the anterior region including what has been referred to as the iridial portion of the primary ciliary processes (Weingeist, 1970), and the middle region, between these two. For clarity, we here refer to these three regions not by structure, but simply by location in the iris-ciliary body, as posterior, anterior and middle, respectively. Our limited analyses of the *pars plana* have given no indication of compositional differences from the slightly more anterior epithelial cells of the outer *pars plicata,* but a full examination of the *pars plana* was precluded by the small number of cells which could be analyzed in this narrow band of thin epithelium.

Sections cut from blocks of the posterior (*pars plicata*) region (Weingeist, 1970) often retained sufficient structure to enable us, in addition, to analyze epithelial cells specifically located at the crests and valleys. We have grouped these analyses from one series of experiments separately for comparison. Since the regular folded structure of the posterior region is not continued into the more anterior convoluted ciliary processes, crests and valleys as such do not exist there. Sections were cut 0.2–0.4 μm in thickness at −80 to −90°C, freeze-dried at 10^{-4} Pa (equivalent to 7.5×10^{-7} Torr), and transferred for analysis to a scanning electron microscope (JEOL JSM 840) equipped with an energy-dispersive spectrometer.

Solutions and Chemicals

The bicarbonate medium contained (in mM): 145 Na^+, 5.9 K^+, 122.1 Cl^-, 15.0 HEPES [4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid], 1.2 Mg^{2+}, 2.5 Ca^{2+}, 1.2 $H_2PO_4^-$, 30 HCO_3^- and 10 glucose at pH 7.30–7.45 and 305–315 mOsmol. Bicarbonate-free solution was prepared by isosmolar replacement of HCO_3^- by Cl^-. Depending on whether or not HCO_3^- was included, the gas bubbled through the solution throughout incubation consisted of either 95%O_2–5%CO_2 or pure O_2, respectively. All chemicals were reagent grade. Bovine albumin (RIA grade, Immuno Chemical Products, Auckland, New Zealand) was dialyzed for 48–60 hr, freeze-dried at −70°C, and stored at 4°C. A 30% (w/v) solution was prepared by dissolving the albumin in the same medium in which the tissue was incubated. Both acetazolamide and bumetanide were added from stock solutions in dimethylformamide. In each case, the same concentration of solvent vehicle (0.1% v/v) was applied to the parallel control preparations.

Data Acquisition and Reduction

The dried sections were imaged with a transmitted electron detector. Measurements were collected with a Tracor Northern 30 mm^2 x-ray detector, using a probe current of 140–200 pA for 100 sec at an accelerating voltage of 20 kV. The intracellular data were obtained by the electron beam scanning a rectangular area within the nucleus of each selected NPE or PE cell which varied from ~0.9 × 1.2 μm to ~2.4 × 3.0 μm depending on the size of the nucleus analyzed. It must be emphasized that a great strength of the electron probe is the capability of directly visualizing those cells and those subcellular targets which are analyzed, thereby ensuring that only the analyzed epithelial cells and not the extracellular compartment or other cellular components contributed data on elemental composition.

The elemental peaks were quantified by filtered least-square fitting to a library of mono-elemental peaks (Bowler, Purves & Macknight, 1991). The library spectra for Na, Mg, Si, P, S, Cl, K and Ca were derived from microcrystals sprayed onto a Formvar film. White counts were summed over the range 4.6–6.0 keV, and corrected for the nontissue contributions arising from the Al specimen holder and Ni grid.

Although we attempted to calculate ion contents and concentrations using the internal standard technique of Dörge et al. (1978) (Bowler et al., 1996; Rick et al., 1982), we have found that we can not obtain consistent data by relying on this approach in the ciliary epithelium. The reasons for this include the presence of residual adherent vitreous, which can limit access of the albumin solution to the aqueous surface of the epithelium, particularly in the *pars plicata* region. Nevertheless, we continued to apply the albumin solution comprising the internal standard (Dorge et al., 1978) in these experiments, since we were concerned that its omission might increase tendencies for the tissue to crumble or fragment during cryosectioning.

As discussed in a previous study (McLaughlin et al., 1998), the Na, K and Cl signals were routinely normalized to the P signal obtained in the same scanned area of each cell, yielding molar ratios of these elements. We have previously estimated the phosphorus content in this tissue to constitute ~500 mmol/kg dry weight (Bowler et al., 1996). Phosphorus was chosen for normalization because of the constancy of the intracellular signal, which almost entirely reflects the covalently linked fraction in epithelial cells. For example, inorganic phosphate (P_i) is accumulated to only 3 mmol/kg intracellular water in the epithelial cells of frog skin (Civan et al., 1983). In such cells, the total pool of ATP, ADP, phosphocreatine and P_i corresponds to only 5% of the total P pool measured in the ciliary epithelial cells (Bowler et al., 1996). The validity of normalizing to P has been experimentally demonstrated by the close linear relationship we have obtained between the two largely intracellular elements, K and P (Fig. 3 of Bowler et al., 1996). The values we report for Na/P, Cl/P and K/P are the measured estimates of the intracellular Na, Cl and K contents, respectively. Although it is impossible to estimate ion concentrations in mmol/liter from these data, the changes in intracellular contents of (Na + K) or of (Na + K + Cl) must reflect changes in intracellular water content (Abraham et al., 1985). For this reason, the sums of the measured ratios (Na/P + K/P + Cl/P) and also (Na/P + K/P − Cl/P) are entered in the Table and cited, where appropriate, in the text. In addition, (Na/P + K/P − Cl/P) reflects changes in the amount of cellular anion other than Cl^-.

The numbers of cells analyzed are indicated by the symbol *n*. Values are presented as the means ± 1 SEM. Differences between groups have been analyzed by ANOVA using nonparametric (Kruskal-Wallis) testing, and the probabilities (*P*) of the null hypothesis have been calculated with the Dunn Multiple Comparisons post-test. The basis for this choice of statistical analysis is discussed in detail elsewhere (Bowler, Purves & Macknight, 1991).

Results

Under all conditions, the contents of Na, K and Cl (normalized as Na/P, Cl/P and K/P, respectively) were consistently greater in NPE than in PE cells. As discussed previously (McLaughlin et al., 1998), these differences in content do not necessarily imply differences in ion concentrations between the two cell-types. Indeed, differences between the cell types in the nature and amount of nondiffusible P-containing organic compounds may well underlie these differences in the ion ratios. Since the patterns of change were the same in the two cell types in all of the experimental protocols examined in the present study, we have focused on the combined data obtained with the equal numbers of NPE and PE cells. These combined data are presented both in the Table and in the figures. In general, analyses of cells from the anterior and middle regions were in agreement with our previous results (McLaughlin et al., 1998).

Incubation without or with Bicarbonate/CO_2 Solution

The Table summarizes the effects of incubation in either bicarbonate-free or bicarbonate/CO_2 solution on the composition for all the ciliary epithelial cells of the three regions. Figure 3 illustrates the effects on Cl/P and K/P.

Table. Comparison of the effects of bicarbonate and bicarbonate-free control solutions on ion content of all cell types in different regions of the rabbit iris ciliary body

A. Incubation in HCO_3^--free solution.

Region	*n*	Ratio (mmol/mmol)				
		Na/P	Cl/P	K/P	(Na + K + Cl)/P	(Na + K − Cl)/P
Anterior (19 sections)	232	0.087 ± 0.003	0.215 ± 0.003	1.031 ± 0.010	1.333 ± 0.011	0.904 ± 0.008
Middle (20 sections)	242	0.100 ± 0.004	0.213 ± 0.003	1.005 ± 0.008	1.318 ± 0.011	0.892 ± 0.007
Posterior (20 sections)	258	0.063 ± 0.003	0.221 ± 0.004	1.158 ± 0.009	1.442 ± 0.012	1.000 ± 0.007
Differences						
Anterior-middle		−0.012 ± 0.005	0.001 ± 0.006	0.026 ± 0.014	0.015 ± 0.019	0.012 ± 0.012
P		NS	NS	NS	NS	NS
Anterior-posterior		0.025 ± 0.005	−0.006 ± 0.006	−0.127 ± 0.014	−0.109 ± 0.019	−0.096 ± 0.011
P		<0.001	NS	<0.001	<0.001	<0.001
Middle-posterior		0.037 ± 0.005	−0.008 ± 0.006	−0.153 ± 0.014	−0.124 ± 0.018	−0.108 ± 0.011
P		<0.001	NS	<0.001	<0.001	<0.001

B. Incubation in HCO_3^- solution.

Region	*n*	Ratio (mmol/mmol)				
		Na/P	Cl/P	K/P	(Na + K + Cl)/P	(Na + K − Cl)/P
Anterior (20 sections)	244	0.106 ± 0.004	0.339 ± 0.006	1.151 ± 0.011	1.596 ± 0.016	0.918 ± 0.009
Middle (18 sections)	218	0.102 ± 0.004	0.309 ± 0.006	1.107 ± 0.011	1.518 ± 0.016	0.901 ± 0.008
Posterior (20 sections)	274	0.094 ± 0.004	0.279 ± 0.004	1.242 ± 0.011	1.615 ± 0.014	1.057 ± 0.009
Differences						
Anterior-middle		0.004 ± 0.005	0.031 ± 0.006	0.044 ± 0.014	0.078 ± 0.019	−0.017 ± 0.012
P		NS	<0.001	<0.05	<0.001	NS
Anterior-posterior		0.013 ± 0.005	0.061 ± 0.006	−0.096 ± 0.014	−0.022 ± 0.018	−0.139 ± 0.011
P		NS	<0.001	<0.001	NS	<0.001
Middle-posterior		0.009 ± 0.005	0.031 ± 0.006	−0.140 ± 0.014	−0.100 ± 0.019	−0.156 ± 0.012
P		NS	<0.001	<0.001	<0.001	<0.001

Data in *A* & *B* from eyes of 5 animals; mean ± SEM
n is total number of cells analyzed from the number of sections shown

C. Differences between incubation in HCO_3^- and HCO_3^- -free solutions.

Region	*n*	Ratio (mmol/mmol)				
		Na/P	Cl/P	K/P	(Na + K + Cl)/P	(Na + K − Cl)/P
Anterior		0.019 ± 0.005	0.125 ± 0.006	0.120 ± 0.014	0.263 ± 0.019	0.014 ± 0.012
P		<0.01	<0.001	<0.001	<0.001	NS
Middle		0.002 ± 0.006	0.096 ± 0.006	0.102 ± 0.014	0.200 ± 0.019	0.009 ± 0.012
P		NS	<0.001	<0.001	<0.001	NS
Posterior		0.030 ± 0.005	0.057 ± 0.006	0.089 ± 0.015	0.176 ± 0.018	0.057 ± 0.011
P		<0.001	<0.001	<0.001	<0.001	<0.001

In bicarbonate-free solution (Table, *A*), the ratio for K/Na contents was higher in the posterior than in the two more anterior regions. This observation is compatible with either a lower relative influx of Na^+ or a greater rate of Na^+/K^+ exchange through the Na^+ pump in the posterior region. Compositions of epithelial cells in the three regions were otherwise similar in the absence of bicarbonate and CO_2.

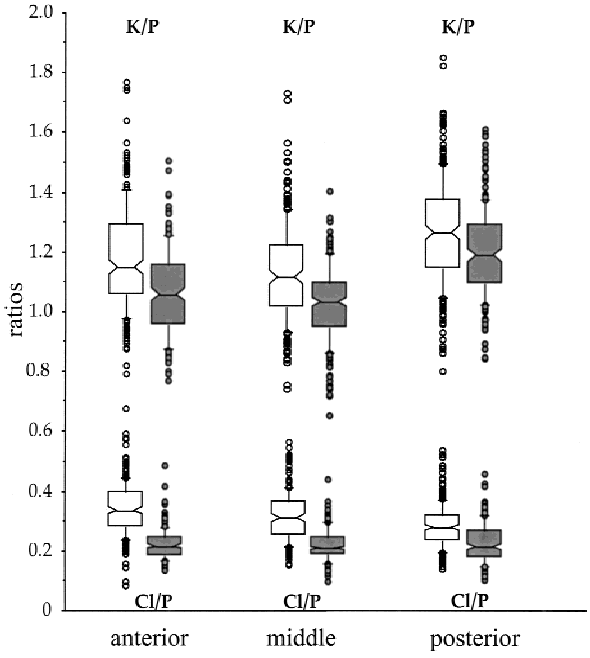

Fig. 3. Effects of incubation in either a bicarbonate/CO_2 or a bicarbonate-free solution on epithelial Cl/P and K/P ratios in the three regions. The medians are indicated by the central horizontal lines, the lower and upper lines include all data between the 25th and 75th percentiles, and the 'whiskers' display the data range between the 10th and 90th percentiles. Circles are individual data points that lie outside of this range. Data were obtained from experiments using eyes from five animals: bicarbonate/CO_2 solution (clear symbols)—anterior region, 244 cells from 20 sections; middle region, 218 cells from 18 sections; posterior region, 268 cells from 20 sections. Bicarbonate-free solution (shaded symbols)—anterior region, 232 cells from 19 sections; middle region, 242 cells from 20 sections; posterior region, 258 cells from 20 sections.

There were no statistically significant differences in Cl/P among the three regions in bicarbonate-free solution. However, in the bicarbonate/CO_2 solution, the Cl/P in the anterior regions was significantly higher than in the middle region ($P < 0.001$), and both these regions had appreciably higher Cl/P ratios than the posterior region ($P < 0.001$).

In bicarbonate-free solution, K/P did not differ significantly between the anterior and middle regions. However, the mean posterior region K/P was significantly higher than in the other regions ($P < 0.001$ for both comparisons). In the bicarbonate/CO_2 solution, the K/P in the anterior region was significantly higher than in the middle region and, as in the bicarbonate-free solution, the mean posterior K/P was significantly higher than both anterior and middle means.

When the effects of incubation with or without bicarbonate/CO_2 are compared (Table, *C*), Cl/P was appreciably lower in tissues incubated in bicarbonate-free solution, as previously reported (Bowler et al., 1996; McLaughlin et al., 1998). This was seen in all three regions, with the greatest differences being in the anterior and middle regions, where Cl/P was decreased by some 58% (anterior) and 45% (middle), respectively. In contrast, the posterior Cl/P, although significantly lower, was decreased only by some 26%. Similar differences were seen with K/P. There was relatively little effect on the Na/P in the anterior and middle regions. However, posterior Na/P decreased appreciably.

The changes in individual ratios are reflected in the data for (Na/P + K/P + Cl/P), which provides a measure of the change in total diffusible ions, and for (Na/P + K/P − Cl/P) which allows estimation of the change in unmeasured cell ionic charge. The significantly lower values for (Na/P + K/P + Cl/P) in bicarbonate-free solution indicate that cell water content was lower under these conditions. The similarities of the values of (Na/P + K/P − Cl/P) of the anterior and middle regions in each solution and between solutions indicates that the total negative charge contributed by other anions was very similar in both regions and that incubation in the bicarbonate-free solution did not result in a lower cell bicarbonate content than incubation in its presence. In both solutions, the posterior region (Na/P + K/P − Cl/P) was appreciably higher than in the more anterior regions, indicating that these cells contained more unmeasured anion. The fact that this difference increased significantly in bicarbonate/CO_2 solution is consistent with an increase in the cell bicarbonate of the posterior region under these conditions.

Incubation in Bicarbonate/CO_2 Solution with Acetazolamide

In a bicarbonate/CO_2-containing solution, acetazolamide (0.5 mM) reduced the Cl/P and K/P ratios towards the levels measured after incubation in bicarbonate-free solution (Fig. 4), as previously reported (McLaughlin et al., 1998). Specifically, in experiments using tissue from 3 animals, the presence of 0.5 mM acetazolamide decreased Cl/P and K/P in the anterior region by 0.106 ± 0.008 ($P < 0.001$) and 0.054 ± 0.018 ($P < 0.05$) respectively. In the middle region Cl/P and K/P fell by 0.107 ± 0.009 ($P < 0.001$) and 0.088 ± 0.019 ($P < 0.001$) respectively. The reduction of Cl/P in the posterior region was smaller (0.041 ± 0.008; $P < 0.001$), as expected from the smaller decrease in chloride following incubation in bicarbonate-free solution, while K/P was actually slightly higher, although the difference was not significant. There were small decreases in Na/P in all regions of 0.013, 0.021 and 0.021 respectively, none of which reached statistical significance.

Incubation in Bicarbonate/CO_2 Solution with Bumetanide

Incubation in a bicarbonate/CO_2 solution containing 0.05 mM bumetanide (Fig. 5) resulted in marked increases in

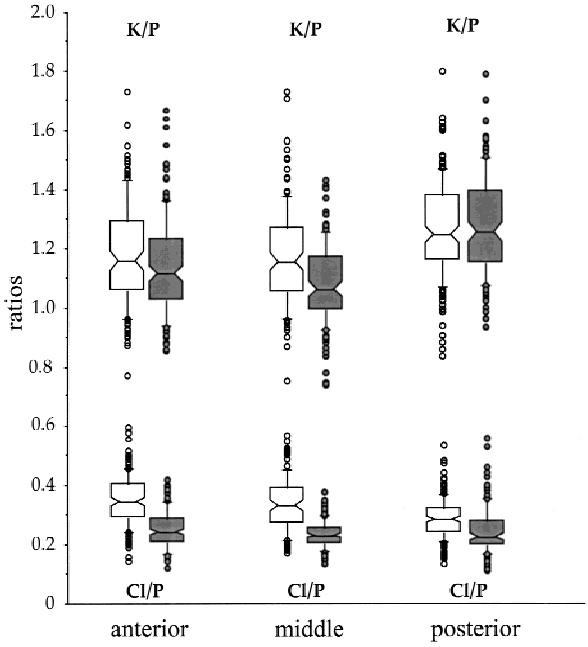

Fig. 4. Effects of acetazolamide (0.5 mM) on ciliary epithelial Cl/P and K/P ratios in a bicarbonate/CO_2 solution. The medians are indicated by the central horizontal lines, the lower and upper lines include all data between the 25th and 75th percentiles, and the 'whiskers' display the data range between the 10th and 90th percentiles. Circles are individual data points that lie outside of this range. Data were obtained from experiments using eyes from three animals: bicarbonate/CO_2 solution (clear symbols)—anterior region, 148 cells from 12 sections; middle region, 122 cells from 10 sections; posterior region, 174 cells from 12 sections. Bicarbonate/CO_2 solution + acetazolamide (shaded symbols)—anterior region, 154 cells from 13 sections; middle region, 148 cells from 12 sections; posterior region, 146 cells from 11 sections.

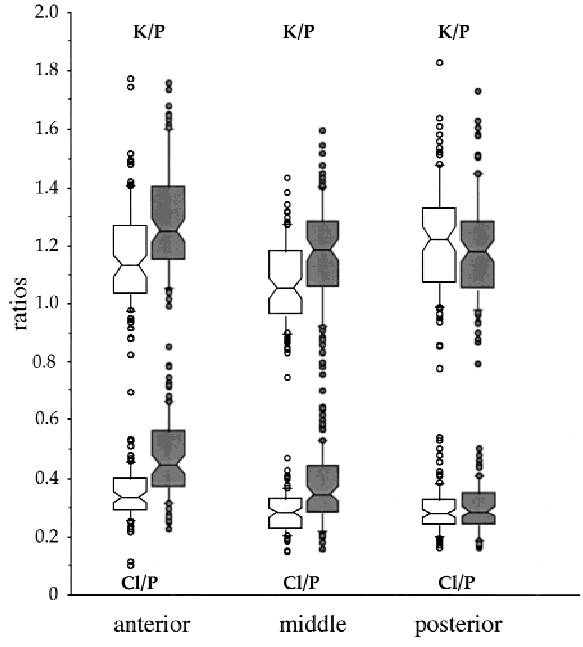

Fig. 5. Effects of bumetanide (0.05 mM) on ciliary epithelial Cl/P and K/P ratios in a bicarbonate/CO_2 solution. The medians are indicated by the central horizontal lines, the lower and upper lines include all data between the 25th and 75th percentiles, and the 'whiskers' display the data range between the 10th and 90th percentiles. Circles are individual data points that lie outside of this range. Data were obtained from experiments using eyes from two animals. Bicarbonate/CO_2 solution (clear symbols)—anterior region, 102 cells from 8 sections; middle region, 96 cells from 8 sections; posterior region, 96 cells from 8 sections. Bicarbonate/CO_2 solution + bumetanide (shaded symbols)—anterior region, 96 cells from 10 sections; middle region, 96 cells from 8 sections; posterior region, 96 cells from 10 sections.

both Cl/P and K/P in the anterior and middle regions, confirming our previous results (McLaughlin et al., 1998). Specifically, in experiments using tissue from 2 animals, the presence of 0.05 mM bumetanide increased Cl/P and K/P in the anterior region by 0.119 ± 0.014 ($P < 0.001$) and 0.127 ± 0.026 ($P < 0.001$) respectively. In the middle region Cl/P and K/P rose by 0.063 ± 0.014 ($P < 0.01$) and 0.063 ± 0.026 ($P < 0.01$) respectively. In contrast, there was no significant effect on either Cl/P or K/P in the posterior region. There were no significant changes in Na/P in any region.

Comparison of Composition of Cells from the Crests and Valleys in the *pars plicata* Region

Only in the posterior, *pars plicata,* region was it possible to make a direct comparison between the cells in the crests and valleys (*see* Methods). In tissues from 4 animals, the values and their differences Δ in bicarbonate-free solution were: Na/P crests ($n = 59$) 0.064, valleys ($n = 60$), 0.064, $\Delta = 0.000 \pm 0.011$; Cl/P crests ($n = 59$) 0.219, valleys ($n = 60$), 0.208, $\Delta = 0.011 \pm 0.011$; K/P crests ($n = 59$) 1.144, valleys ($n = 60$), 1.171, $\Delta = 0.027 \pm 0.025$. The values in bicarbonate/CO_2 solution were: Na/P crests ($n = 46$) 0.089, valleys ($n = 38$), 0.091, $\Delta = 0.002 \pm 0.013$; Cl/P crests ($n = 46$) 0.319, valleys ($n = 38$), 0.299, $\Delta = 0.020 \pm 0.016$; K/P crests ($n = 46$) 1.283, valleys ($n = 38$), 1.330, $\Delta = 0.047 \pm 0.039$. In neither situation were any of these small differences statistically significant.

Discussion

The absence of detectable differences in diffusable elemental content between cells in the crests and valleys of the *pars plicata* has two implications. First, unstirred layers played insignificant roles. Otherwise, the elemental composition of cells in the more accessible crests and less accessible valleys should have been different. Second, the cells in the crests and valleys evidently possess similar intrinsic ion transport capacities.

In contrast to the functional homogeneity noted in

planes *perpendicular* to the antero-posterior axis of the eye, substantial heterogeneity was evident *along* the antero-posterior axis. Significant differences were noted in the response of the Cl content to changes in HCO_3^-/CO_2 concentration, acetazolamide and bumetanide. Each of these perturbations is expected to alter ciliary epithelial secretion (Krupin & Civan, 1995), and each alters the elemental composition of the ciliary epithelium, as we have previously reported (McLaughlin et al., 1998). The present study documents that each perturbation exerted greater effects on the anterior than on the posterior epithelium.

The fall in Cl content caused by either omitting HCO_3^-/CO_2 or inhibiting carbonic anhydrase with acetazolamide (in the presence of HCO_3^-/CO_2) was entirely expected; in both cases, the rate of HCO_3^- delivery to the AE2 Cl^-/HCO_3^- exchanger of the PE cells was thereby slowed. However, the basis for the increase in Cl content triggered by bumetanide (again in the presence of HCO_3^-/CO_2) was initially unclear because the Na^+-K^+-$2Cl^-$ symport can certainly underlie cellular uptake of solute under many conditions (Hoffmann, 1987). In the absence of HCO_3^-/CO_2, bumetanide indeed reduces the Cl/P ratio in the ciliary epithelium (McLaughlin et al., 1998). We have suggested that the availability of HCO_3^- permits the AE2-type HCO_3^-/Cl^- exchanger of the pigmented ciliary epithelial cells (Counillon et al., 2000) to accumulate Cl^- above the equilibrium value for the Na^+-K^+-$2Cl^-$ symport; under these conditions, inhibiting the symport with bumetanide will reduce the rate of solute efflux through this pathway, elevating the Cl content. We cannot verify this conclusion directly in the absence of measurements of intracellular concentration, rather than content. However, based on calculations of the net thermodynamic driving force as a function of Cl^- concentration within the ciliary epithelial cells, intracellular Cl^- concentrations below ~50 mM should favor solute uptake, and concentrations above that value should drive solute release out of the cells through the symport (Fig. 6 of McLaughlin et al., 1998). This bidirectionality of transfer by the symport is consistent with the appearance of bumetanide-inhibitable ^{86}Rb efflux from ouabain-treated, rabbit NPE cells (Dong & Delamere, 1994). In another preparation, the light-induced reduction in $[K^+]$ from 5 to 2 mM in the subretinal space can also trigger a physiologic reversal in the net driving force on the Na^+-K^+-$2Cl^-$ symport of the apical membrane of retinal pigment epithelial cells (Bialek & Miller, 1994). Net movement through the symport out of the cell has also been postulated to be involved in secretion of cerebrospinal fluid (Keep, Xiang & Betz, 1994).

The present data do not permit us to identify with certainty the basis for the reduced responsiveness of the posterior epithelium to all three perturbations: omitting HCO_3^-/CO_2, inhibiting carbonic anhydrase with acetazolamide and inhibiting the symport with bumetanide. However, we note that there was a smaller decrease in content of diffusible cation (Na/P + K/P) in all regions in the nominal absence of HCO_3^-/CO_2. Nearly all the increase in diffusible cation content produced by including HCO_3^-/CO_2 in the bath was balanced by an increase in Cl content in the anterior regions, whereas about half was balanced by an increase in unmeasured anion content [(Na + K – Cl)/P] in the posterior region. It is reasonable to presume that the gain in unmeasured anion produced by adding HCO_3^-/CO_2 reflected an elevation of cell HCO_3^-. If inclusion of HCO_3^-/CO_2 accelerates the rate of delivery of cell HCO_3^- to the Cl^-/HCO_3^- exchangers to comparable degrees throughout the epithelium, we must conclude that Cl^-/HCO_3^- turnover is slower in the posterior (*pars plicata*) than in the anterior epithelium. It is interesting that the ratio of K to Na content was higher in the posterior than in the anterior epithelium. This is consistent with a lower turnover of $Na^+$and K^+ in the posterior region. A slower turnover of Cl^-/HCO_3^- exchange in the posterior epithelium would also be consistent with the reduced responsiveness to bumetanide. If the posterior epithelium only accumulates Cl^- close to the equilibrium level for Na^+-K^+-$2Cl^-$ cotransport (rather than above that level, as postulated for the anterior epithelium), bumetanide's inhibition of the symport would have no detectable effect.

The implications of our findings for human aqueous dynamics are as yet uncertain. Given the importance of Cl^- as the major anion of the aqueous humor, we have taken changes in intracellular Cl content as an index of response to experimental perturbations known to affect ciliary epithelial secretion, particularly external HCO_3^- concentration and application of acetazolamide (Krupin & Civan, 1995). Carbonic anhydrase inhibitors both inhibit accumulation of Cl in the ciliary epithelium (McLaughlin et al., 1998) and reduce aqueous humor formation. The reduction in Cl content is much greater in the anterior than the posterior epithelium. Therefore, the data are consistent with the suggestion that the anterior part of the ciliary processes is the major site of aqueous humor secretion (Ghosh et al., 1991; Coca-Prados & Sanchez-Torres, 1998; Dunn et al., 2001). It is also plausible, as Coca-Prados et al. suggested, (Ghosh et al., 1991), that the posterior ciliary epithelium can serve as a reabsorptive structure. Although the small responses to medium HCO_3^-/CO_2 and to acetazoleamide, and the lack of response to bumetanide indicate relatively little Cl^-/HCO_3^- antiport and Na^+-K^+-$2Cl^-$ symport activity in this region, other transporters, as illustrated in Fig. 1*B*, have been identified, which could support reabsorption here. The smaller increase in Cl content and comparable increase in cell volume (monitored by Na/P + K/P) after incubation in HCO_3^-/CO_2 medium as compared to HCO_3^--free medium in the *pars*

plicata suggest a lower intracellular Cl^- concentration posteriorly. Thus, the driving force for Cl^- uptake from the aqueous humor into the NPE cells would be more favorable in the posterior than in the anterior regions of the ciliary epithelium, through the Na^+-K^+-$2Cl^-$ symport (Civan et al., 1996) or other uptake mechanism. Whether or not this region actually supports net reabsorption as illustrated by elements of Fig. 1*B* remains to be demonstrated.

Following initial submission of this report of our functional results, Dunn, Lyttle and Crook (2001) published a complementary study of the anatomical distribution of the Na^+-K^+-$2Cl^-$ symport in the bovine ciliary epithelium. In their careful study, Dunn, Lyttle and Crook observed more symport protein in the PE than in the NPE cells of the *pars plicata* of 1–4 day-old calves. Protein immunofluorescence was most intense along the basolateral surfaces of the PE cells in that region, while the immunofluorescence was more diffuse in the NPE cells. These observations are consistent with functional indications that the symport, when thermodynamically directed inwards, can support uptake of solute by PE cells from the stroma (Wiederholt & Zadunaisky, 1986; Edelman, Sachs & Adorante, 1994; Do & To, 2000; To et al., 2001; Crook et al., 2000). In addition, the same authors found a higher symport content in the NPE than in the PE cells of the *pars plana.* This second observation is consistent with the possibility that a fraction of the NPE cotransporter is incorporated in the basolateral membranes, accounting for the bumetanide-sensitive solute uptake reported previously both by the same group (Crook et al., 1992; Von Brauchitsch & Crook, 1993; Crook & Riese, 1996; Riese et al., 1998) and others (Dong & Delamere, 1994; Civan et al., 1996). Among other results, Dunn, Lyttle and Crook (2001) also reported a third intriguing observation, that the symport distribution was far more variable in adult cows than in 1–4 day-old calves. In contrast to the uniform results obtained with the young calves, 60% of the adult tissues displayed either cytoplasmic and punctate or greatly diminished basolateral immunofluorescence. In these animals, fluorescence was reported to be either the same or slightly greater in NPE than in PE cells. The present functional results were obtained with adult rabbits, presumably more relevant to the adult than to the young calf data of Dunn, Lyttle and Crook (2001). Thus, their anatomical data are not inconsistent with the model of Fig. 1, suggesting that the the Na^+-K^+-$2Cl^-$ cotransporter may underlie solute transfer across the basolateral membranes of both the PE and NPE cells in different regions of the ciliary epithelium.

Supported in part by a Project Grant from the Health Research Council of New Zealand and Lottery Health and by Research Grants EY08343 and EY01583 from the National Institutes of Health, USA.

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