Electron microprobe analysis of rabbit ciliary epithelium indicates enhanced secretion posteriorly and enhanced absorption anteriorly

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McLaughlin CW, Zellhuber-McMillan S, Macknight AD, Civan MM. Electron microprobe analysis of rabbit ciliary epithelium indicates enhanced secretion posteriorly and enhanced absorption anteriorly. Am J Physiol Cell Physiol 293: C1455–C1466, 2007. First published August 29, 2007; doi:10.1152/ajpcell.00205.2007.—The rate of aqueous humor formation sequentially across the pigmented (PE) and nonpigmented (NPE) ciliary epithelial cell layers may not be uniform over the epithelial surface. Because of the tissue’s small size and complex geometry, this possibility cannot be readily tested by conventional techniques. Rabbit iris-ciliary bodies were divided, incubated, quick-frozen, cryosectioned, and freeze-dried for electron probe X-ray microanalysis of the elemental contents of the PE and NPE cells. We confirmed that preincubation with ouabain to block Na+/K+-ATPase increases Na+ and decreases K+ contents far more anteriorly than posteriorly. The anterior and posterior regions were the iridial portion of the primary ciliary processes and the pars plicata, respectively. Following interruption of gap junctions with heptanol, ouabain produced smaller changes in anterior PE cells, possibly reflecting higher Na+ or K+ permeability of anterior NPE cells. Inhibiting Na+ entry selectively with amiloride, benzamil, or dimethylamiloride reduced anterior effects of ouabain by ~50%. Regional dependence of net secretion was also assessed with hypotonic stress, which stimulates ciliary epithelial cell regulatory volume decrease (RVD) and net Cl− secretion. In contrast to ouabain’s actions, the RVD was far more marked posteriorly than anteriorly. These results suggest that 1) enhanced Na+ reabsorption anteriorly, likely through Na+/H+ exchange, mediates the regional dependence of ouabain’s actions; and 2) secretion may proceed primarily posteriorly, with secondary processing and reabsorption anteriorly. Stimulation of anterior reabsorption might provide a novel strategy for reducing net secretion.

aqueous humor; rabbit iris-ciliary body; net secretion; sodium channels; sodium/hydrogen ion exchange

THE INTRAOCULAR PRESSURE (IOP) is directly dependent on the rate of inflow of aqueous humor and the resistance to its outflow. Reducing IOP is a major strategy in the pharmacological treatment of glaucoma. Secrecion of aqueous humor arises primarily from ciliary epithelial transfer of NaCl from the stroma of the ciliary processes to the posterior chamber of the eye, establishing an osmotic driving force for secondary water transport. Unidirectional NaCl secretion is considered to proceed in three steps (Fig. 1A): 1) electroneutral uptake of Na+ and Cl− by the pigmented ciliary epithelial (PE) cells through antiports and symports, 2) NaCl transfer to nonpigmented ciliary epithelial (NPE) cells through gap junctions, and 3) release of Cl− and Na+ to the aqueous humor largely through Cl− channels and Na+,K+-activated ATPase, respectively. Multiple mechanisms also subserve recycling of Na+ and K+ at the aqueous (5, 10–12, 15, 42, 47) and stromal surfaces (14, 36) (Fig. 1B), providing a functional basis for potential net reabsorption, as yet undocumented.

Several lines of evidence have suggested that secretion across the ciliary epithelium is not uniform and that the maximal net secretion possibly proceeds across the anterior region, raising the possibility of novel therapeutic strategies (see DISCUSSION). Inconsistent with this view is the report that ouabain application reveals reabsorption of Na+ at the aqueous surface that is maximal anteriorly (33). In the current work, we have addressed this issue in two ways. First, we have reexamined whether reabsorption of Na+ is indeed maximal in the anterior region and whether that reentry can be blocked by inhibiting Na+ channels and Na+/H+ exchange. Second, we have exploited the recent observation that hypotonic swelling of the entire ciliary epithelium both triggers a regulatory volume decrease (RVD) and stimulates net Cl− secretion with identical time courses (13). This has permitted us to scan the ciliary epithelium, using the RVD as an index of region-specific net secretion.

METHODS

The methods used in this study for tissue freezing, cryosectioning, and analysis have been described in detail elsewhere (3, 31, 33). Cellular model. Dutch black-belted rabbits of either sex and at least 6 wk postweaning were provided by the Department of Laboratory Animal Sciences, University of Otago Medical School. Animals were treated in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research, and protocols were approved by both the Otago University Animal Ethics Committee (ET16/04) at Dunedin, New Zealand, the site of the experimental work, and the Institutional Animal Care and Use Committee of the University of Pennsylvania (A3079-01). Following anesthesia with 30 mg/kg pentobarbital sodium, rabbits were killed by injecting air into the marginal ear vein. The iris-ciliary body (ICB) was excised from each enucleated eye and cut into quarters, and each quarter was bonded with cyanoacrylate to a Mylar support frame on its stromal border. Quadrants of each ICB were mounted between the two halves of incubation chambers, so oriented as to occlude the common aperture.

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Each half-chamber was filled with 1.5 ml, with fresh solution constantly infused at 0.5 ml/min. The flow rates were higher during solution changes. Aspiration of excess solution maintained equal levels of fluid on each side of the epithelium, with no pressure gradient. A gas lift in each half-chamber aerated and gently stirred the solution. Drugs were added to both sides of the tissue to maximize the effects. We have previously noted that ouabain produces larger changes in elemental composition when added to both aqueous and stromal surfaces than to one side alone.

Tissues were incubated for 1–2 h at room temperature (18–22°C) initially under control conditions. Pairs of quadrants (one from each eye) were then incubated for at least 30 min under either control or experimental conditions. After incubation, the tissues were blotted and then plunged into liquid propane at −180°C to freeze the preparation rapidly to preclude redistribution of ions and water. Blocks were fractured from the frozen tissue under a dissecting microscope (×7). Care was paid to the origin and orientation of the block. Thus, after transfer of a block to the cryoultramicrotome and subsequent trimming, we could identify and accurately select the region from which the sections were cut. Sections were usually cut first from the pupillary side and perpendicular to the major plane of the ICB. The orientation of the block was then reversed, and perpendicular sections were taken from the side of the pars plicata. The sections were cut 0.4–0.6 μm thick at −90 to −100°C, freeze-dried at 10⁻⁴ Pa (7.5 × 10⁻⁷ Torr), and transferred for analysis to a scanning electron microscope (JEOL JSM 840) equipped with an energy-dispersive X-ray spectrometer.

We analyzed two well-defined regions, the posterior and anterior portions of the ciliary epithelium. The posterior region, the posterior pars plicata, displays long ciliary processes reaching regularly down to the iris. In contrast, the anterior region, comprising the iridal portion of the primary ciliary processes (48), expresses irregularly shaped cross-sectional areas. The anterior folds do not necessarily extend to the iris in each section and can reach the iris in an earlier or subsequent section.

Fig. 1. Pathways for unidirectional secretion (A) and possible reabsorption (B) across the ciliary epithelium. PE, pigmented ciliary epithelial cells; NPE, nonpigmented ciliary epithelial cells.

Fig. 2. X-ray spectra collected during irradiation of single visualized intracellular sites in NPE cells from anterior ciliary epithelium under control conditions (A), following incubation with 100 μM ouabain alone for 30 min (B), and following incubation with 100 μM ouabain 40–60 min before incubation with 100 μM ouabain for 30 min (C). The spectra were chosen to be representative of the averaged findings presented in Figs. 3, 5, and 6.
Solutions and chemicals. The isotonic solutions contained (in mM) 145 \( \text{Na}^+ \), 5.9 \( \text{K}^+ \), 122.1 \( \text{Cl}^- \), 15.0 HEPES, 1.2 \( \text{Mg}^{2+} \), 2.5 \( \text{Ca}^{2+} \), 1.2 \( \text{H}_2\text{PO}_4^- \), 30 \( \text{HCO}_3^- \) and 10 glucose at pH 7.30 – 7.45 and 305 – 315 mosmol/kg \( \text{H}_2\text{O} \). Hypotonic solutions (175 mosmol/kg \( \text{H}_2\text{O} \)) were usually comparably higher in NPE than PE cells. In the absence of direct measurements of water content, the normalized anion gap, defined as \( \left( \frac{\text{Na}^+ + \text{K}^+}{\text{Cl}^-} \right) / \text{P} \), was used as an approximate index of changes in intracellular water content (34), although other unmeasured anions can also contribute to this parameter.

Data acquisition and reduction. Electron probe X-ray microanalysis permits both quantification and localization of intracellular elements within visualized NPE or PE cells. Using an electron microscope, we targeted a specific area within each cell. Electron-beam irradiation ionizes a small fraction of the atoms bombarded. After an electron is displaced from an inner atomic shell, an outer shell electron can take its place. Electron relaxation from a higher to a lower energy state releases a quantum of X-ray energy. Measurement of the number of quanta at each characteristic energy permits quantification of the identified intracellular elements.

The freeze-dried sections were visualized with a transmitted electron detector. X-rays were collected with a Tracor Northern 30-mm\(^2\), energy-dispersive X-ray detector, with a probe current of 140 – 200 pA and an accelerating voltage of 20 kV. The intracellular data were obtained by scanning the electron beam over a rectangular area within the nucleus of each selected cell. The nucleus is chosen to avoid any possible contribution from extracellular \( \text{Na}^+ \) and \( \text{Cl}^- \) that might be significant at intracellular sites closer to the plasma membrane. Direct measurements of Chironomus salivary gland cells have demonstrated that the intracellular activities of \( \text{K}^+ \) and \( \text{Cl}^- \) are the same in the nucleoplasm and cytosol (38, 39). The dimensions of the irradiated areas varied from \( \sim 0.9 \times 1.2 \) to \( \sim 2.4 \times 3.0 \) \( \mu \)m, depending on the size of the nucleus analyzed. PE and NPE cells were analyzed in pairs, to enable the cellular compositions of the PE and NPE layers to be compared on an individual cell pair basis.

Elemental peaks were quantified by filtered least-square fitting to a library of monoelemental peaks (3). The library spectra for \( \text{Na}, \text{Mg}, \text{Si}, \text{P}, \text{S}, \text{Cl}, \text{K}, \text{and} \text{Ca} \) were derived from microcrystals sprayed onto a Formvar film. In addition to the quantal element-specific X-rays, irradiating sections with an electron beam produces nonquantal continuous or white radiation, reflecting electron deceleration by coulombic interaction with atomic nuclei. The white counts (w), an index of tissue mass (4), were summed over the range 4.6 – 6.0 keV and corrected for the nonissue contributions arising from the aluminum specimen holder and nickel grid. As discussed previously (31), \( \text{Na}, \text{K}, \text{and} \text{Cl} \) signals were normalized to the P signal obtained in the same scanned area of each cell, yielding molar ratios of these elements. The mean P content of the tissue is \( \sim 500 \) mmol/kg dry wt (2). Phosphorus is chosen for normalization because of the constancy of its intracellular signal, almost entirely reflecting the covalently linked fraction in epithelial cells (e.g., Ref. 4). Normalization to P has been validated by the observed close linear relationship linking the two largely intracellular elements \( \text{K} \) and \( \text{P} \) (Fig. 3 in Ref. 2). NPE cells have the same P content anteriorly and posteriorly, as is true for the PE cells (Table 1 in Ref. 33). In that table, the dry weight content of P was \( \sim 12\% \) lower in NPE than in PE cells in both regions so that the ion contents normalized to P were usually comparably higher in NPE than PE cells. This observation was confirmed in the present work. In 4,288 analyses of NPE and PE cells anteriorly and posteriorly, the P content was \( 11.9 \pm 0.6\% \) higher in the PE cells.

In the absence of direct measurements of water content, the normalized ion contents do not provide a direct estimate of intracellular ion concentrations. However, the sum of the normalized contents of Na and K \( ([\text{Na} + \text{K}] / \text{P}) \) is a useful index of intracellular water content (1). Likewise, we have used the normalized anion gap, defined as \( ([\text{Na} + \text{K}] - \text{Cl}] / \text{P} \), as an approximate index of changes in intracellular \( \text{HCO}_3^- \) content (34), although other unmeasured anions can also contribute to this parameter.
Statistics. Unless otherwise stated, the probability (P) of the null hypothesis has been estimated using Student’s t-test, with significance defined as \( P < 0.05 \). For relatively large numbers of samples, as in the present work, the t-test is highly robust even in the presence of deviations from a normal distribution (23, 44). The significance of differences among multiple means (Figs. 6 and 7) has been estimated using one-way ANOVA, with the Bonferroni test.

RESULTS

Control spectra. Figure 2 displays representative raw spectra collected by irradiating intracellular PE sites within the anterior ciliary epithelium following incubation under control (A) and experimental conditions (B and C). The signals reflecting intracellular Na, P, S, Cl, and K are identified. The aluminum signal arises from the specimen holder, and not from the tissue. Under control conditions, the ratio of the integrated areas for K to Na is very large. The measured elemental compositions under control and ouabain-treated conditions are presented in Table 1. From those data, the molar ratio of K to Na anteriorly is calculated to be \( \frac{10.1 \pm 0.5}{9.8 \pm 0.5} \) for the NPE and 10.1 ± 0.5 for the PE cells; \( n = 227 \). The K-to-Na content was even higher in the posterior ciliary epithelium (17.0 ± 1.1 for the NPE and 19.0 ± 1.2 for the PE cells; \( n = 226 \)), approximately twice as large as anteriorly (\( P < 0.001 \)). This observation might reflect either a relatively higher rate of extracellular Na\(^+\) entry into the anterior epithelium or a relatively higher rate of Na\(^+\),K\(^+\)-activated ATPase activity posteriorly.

As noted in Data acquisition and reduction in METHODS, the elemental contents normalized to the P signal are usually slightly higher (by \(~12\%)\) in the NPE than PE cells. This observation held in the present work (Table 1) as well for Na/P, Cl/P, and K/P and is at least partly ascribable to the lower P content of the NPE cells (METHODS).

Incubation with ouabain alone. Figures 2 and 3 and Table 1 present the effects of incubation with 100 \( \mu \)M ouabain for 30 min on the elemental contents. Comparison of Fig. 2, A and B, illustrates that inhibition of Na\(^+\),K\(^+\)-activated ATPase with ouabain markedly reduced the K signal and enormously increased the Na signal (\( P < 0.001 \)). Under the current experimental conditions, the Cl signal was largely unchanged (Fig. 3 and Table 1). More prolonged exposure to ouabain leads to entry of Cl\(^-\) together with Na\(^+\) into the cells (33). In the present study, the reduction in K/P was \(~0.4\) posteriorly, slightly less than one-half the fall in K/P noted anteriorly (Fig. 3 and Table 1, \( P < 0.001 \)). The loss of K content was largely balanced by a rise in Na/P but also by a small fall in unmeasured anion [(Na + K – Cl)/P], which may largely reflect HCO\(_3\)-loss. Both anteriorly and posteriorly, ouabain produced cell shrinkage, as monitored by (Na + K)/P. All ouabain-triggered changes in composition were substantially greater in...
the anterior than posterior region (Table 1), confirming our
earlier observation (33).

**Incubation with heptanol and ouabain.** After gap-junctional
communication was interrupted by preincubation with 3 mM
heptanol, incubation with 100 μM ouabain for 30 min still
produced substantially greater changes in the anterior than in
the posterior epithelium (Fig. 4). Under the conditions of the
present work, the ouabain-triggered absolute changes in ele-
mental contents were relatively unchanged by heptanol in the
posterior NPE and PE cells and in the anterior NPE cells.
However, in the anterior PE cells, heptanol reduced the
ouabain-triggered gain in Na/P by 39 ± 5% (P < 0.001) and
loss in K/P by 41 ± 4% (P < 0.001). This phenomenon
could reflect either enhanced Na+ or K+ permeability across
the basolateral membranes of these cells (the anterior NPE
cells).

**Incubation with ouabain and amiloride analogs.** The rate of
Na+ entry can limit the rate at which ouabain changes intra-
cellular ionic composition (30). Published evidence suggests
that NPE cells can take up Na+ through Na+/H+ antiports and
an amiloride-sensitive Na+ channel (5, 6, 35). To test whether
inhibiting these ports of Na+ entry might slow the effects of
ouabain, we incubated tissue quadrants from the same eyes
with or without amiloride before and during incubation with
ouabain (Fig. 5). Once again, the actions of ouabain on Na/P
and K/P were much greater anteriorly than posteriorly.

Amiloride partially prevented these changes where ouabain
otherwise exerted its greater effect, anteriorly. The mean
control values for anterior Na/P and K/P are ~0.1 and
1.1–1.2 (Table 1) so that amiloride reduced the action of
ouabain by ~50% anteriorly in the experiment of Fig. 5
(P < 0.001).

Additional studies were conducted with amiloride, as well as
with the analogs benzamil and dimethylamiloride, in testing
whether inhibition of Na+ entry delays the ouabain-triggered
changes in intracellular composition. The results in Figs. 6 and
7 obtained with the anterior and posterior epithelium, respect-
ively, were averaged from different tissue samples. Benzamil
is relatively specific for inhibiting epithelial Na+ channels,
whereas dimethylamiloride more selectively targets Na+/H+
antiports (27). Both analogs, as well as the parent compound
amiloride, partially inhibited the ouabain-induced changes in
intracellular composition.

All three drugs significantly reduced the gain in Na and loss
of K content in both NPE and PE cells of the anterior region
(Fig. 6, P < 0.05 by one-way ANOVA). Although the ouabain-
triggered gain in Cl/P was not significant at the 0.05 probability
level (Table 1), exposure to amiloride, dimethylamiloride, and
benzamil all reduced Cl/P of ouabain-treated cells with respect
to both the ouabain-exposed and control cells (Fig. 6, P < 0.05
by one-way ANOVA). Amiloride and its two analogs did not

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**Fig. 4.** Effects of interrupting gap junctions with 3.0 mM heptanol on ouabain-produced changes in NPE and PE cell ionic contents. In contrast to the results of Fig. 3, ouabain affected the anterior PE cells far less than the anterior NPE cells. The normalized Na, K, and Cl contents are presented in A, B, and C, respectively. The normalized (Na + K – Cl) contents in D provide an index of HCO3− content. Tissues bathed with heptanol alone (control + heptanol) were obtained from 6 rabbits: 188 PE/NPE cell pairs of 17 sections in the posterior region, and 175 PE/NPE cell pairs of 22 sections in the anterior region. Data obtained after ouabain exposure (ouabain + heptanol) were from 5 animals: 157 PE/NPE cell pairs of 16 sections in the posterior region, and 169 PE/NPE cell pairs of 22 sections in the anterior region. *P < 0.05; ***P < 0.001 compared with control (t-test).
significantly alter the ouabain-triggered loss in unmeasured anion [(Na\(^+\) + K\(^+\) – Cl\(^−\))/P; Fig. 6].

The effects of the drugs on the posterior region were less striking, but both amiloride and dimethylamiloride significantly inhibited the ouabain-triggered uptake of Na\(^+\) and release of K\(^+\) in the NPE cells (Fig. 7, \(P < 0.05\) by one-way ANOVA). As in the anterior region, amiloride, dimethylamiloride, and benzamil all reduced Cl/P of ouabain-treated NPE cells (Fig. 7, \(P < 0.05\) by one-way ANOVA). Amiloride and dimethylamiloride also significantly reduced Cl/P of ouabain-treated PE cells, but the protective effect of benzamil alone was not significant at the 0.05 probability level. As in the anterior region, the three drugs did not significantly affect the ouabain-triggered loss in unmeasured anion [(Na\(^+\) + K\(^+\) – Cl\(^−\))/P; Fig. 7] of NPE and PE cells posteriorly. Interestingly, the inhibitions by dimethylamiloride and benzamil did not appear to be additive, either anteriorly or posteriorly.

**Incubation with dimethylamiloride under baseline conditions.** Dimethylamiloride partially blocked the ouabain-triggered changes in ionic composition, but it was unclear whether the inhibitor would exert any region-specific change in baseline composition. Work in our laboratory (32) previously showed that dimethylamiloride produced small reductions in K/P and Cl/P without significantly altering Na/P, but those measurements were conducted before the region dependence of ciliary epithelial transport was appreciated. To examine this point, we analyzed additional tissues following incubation with or without 50 \(\mu\)M dimethylamiloride in control isosmotic solutions in beakers, to conform more closely to the earlier dimethylamiloride experiments (32).

In the anterior ciliary epithelium, dimethylamiloride reduced K/P, Cl/P, and volume of the NPE cells and increased Na/P slightly. Summarizing the responses on baseline, ouabain-untreated cells, dimethylamiloride tended to trigger a loss of K, matched by a reduction in Cl and unmeasured anion content, presumably HCO\(_3^−\), and cell volume. As indicated in Table 2, some of these effects were significantly greater in the anterior ciliary epithelium. Changes in Na/P were minimal, possibly because of secondary changes in turnover of Na\(^+\)-K\(^+\)-activated ATPase, K\(^+\) channels, and Na\(^+\)-K\(^+\)-2Cl\(^−\) symports, among other transporters.

**Regulatory volume decrease.** The results obtained with ouabain suggested that the anterior region of the intact ciliary epithelium displays substantial reabsorption of Na\(^+\) across the aqueous surface. We also wanted to assess the regional dependence of a stimulation of secretion known to be associated with readily measurable changes in elemental composition. For this purpose, hypotonic swelling of the ciliary epithelium was...
highly suitable. Hypotonic challenge is known to trigger swelling activation of Cl⁻/H⁺ channels in isolated NPE cells and to stimulate net Cl⁻ secretion across intact rabbit ciliary epithelium (13).

Figure 8 presents the elemental compositions of NPE and PE cells in quadrants of ciliary epithelia incubated only in isotonic solution (t = 0 min) and after hypotonic challenge for 5, 20, and 40 min. The hypotonicity triggered progressive reductions in K/P, Cl/P, and volume, monitored as (Na⁺/K⁺)/Cl⁻ in D provide an index of HCO₃⁻ content. Data were obtained from the posterior region of the same sections from 7 animals. Control data were from 226 PE/NPE cell pairs from 26 sections. Data for ouabain treatment alone were from 196 PE/NPE cell pairs from 25 sections. Data for both amiloride and ouabain treatment were from 149 PE/NPE cell pairs from 22 sections. Data for benzamil and ouabain treatment were from 131 PE/NPE cell pairs from 13 sections. Data for benzamil, DMA, and ouabain treatment were from 52 PE/NPE cell pairs from 8 sections. Data for benzamil and DMA treatment were from 64 PE/NPE cell pairs from 8 sections. *P < 0.05 compared with cells exposed to ouabain alone (Ref). Significance was estimated by ANOVA, using the Bonferroni multiple comparisons test.

DISCUSSION

Proteins and biologically active peptides are known to be expressed nonuniformly in different regions of the ciliary epithelium (17–22, 24, 25). One example is the higher expression of α₁/α₂/α₃/β₁/β₂-isofoms of Na⁺,K⁺-activated ATPase in the NPE cells in the anterior than in the posterior region of young calves (7). Another example is the localization of the Na⁺⁺⁺⁺Cl⁻ cotransporter largely at the basolateral edge of the PE cell layer in the anterior region of young calves (17). The possibility has been raised that such nonuniformities might have functional implications (25). However, the small size and structural complexity of the epithelium had precluded progress in testing that proposition experimentally.

We have been exploiting electron-probe X-ray microanalysis (16, 26, 41) to assess the functional topography of the intact ciliary epithelium. This technique permits elemental content measurement of PE and NPE cells in different regions of the rabbit ciliary epithelium (2, 31). Previous microprobe analyses have indicated that the anterior ciliary epithelial cells display more rapid turnover of intracellular Cl⁻ following removal of HCO₃⁻/CO₂ or exposure to the inhibitors bumetanide or acetazolamide (34). The present measurements confirm (33) more
rapid ouabain-induced turnover of intracellular Na\(^+\) and K\(^+\) in the same anterior region. However, whether or not the increased turnover of Na\(^+\), K\(^+\), and Cl\(^-\) anteriorly under certain experimental conditions indeed reflects higher secretory rates by that region, as has been suggested (25, 34), has been unclear. We have addressed this uncertainty in two ways. First, we have reexamined whether exposure to ouabain might possibly trigger/expose solute recycling or reabsorption, rather than secretion, at the anterior aqueous surface. Second, we have built on recent information indicating that hypotonic challenge stimulates net secretion across some or all of the ciliary epithelium (13).

Effects of ouabain. We initially verified that incubation with 100 \(\mu\)M ouabain for 30 min produces changes in Na and K contents twice as large anteriorly than posteriorly. Under the current experimental conditions, interruption of gap-junctional communication with heptanol did not alter the magnitudes of the ouabain-induced exchange of Na\(^+\)/K\(^+\) in the posterior NPE and PE cells and in the anterior NPE cells. In contrast, isolating the anterior PE cells with heptanol reduced the effects of ouabain by \(\approx 50\%\) (cf. Figs. 3 and 4). We conclude that the NPE cells must provide either a substantial source for Na\(^+\) delivery to, or for K\(^+\) removal from, the anterior PE cells. The actions of amiloride and its analogs suggest that it is the rate of Na\(^+\) entry that limits the Na\(^+\)-K\(^+\) exchange. Consistent with studies in other tissues (30), ouabain does not reduce K\(^+\) content until Na\(^+\) enters to maintain electroneutrality. Inhibition of Na\(^+\) entry reduced the anterior, ouabain-produced Na\(^+\)-K\(^+\) exchange by \(\approx 50\%\) (Figs. 5 and 6). It is interesting that simultaneous application of benzamil and dimethylamiloride did not result in additive inhibition, although the former was more effective against the ENaC epithelial Na\(^+\) channel and the latter against Na\(^+\)/H\(^+\) exchange (27). ENaC is known to be expressed by human ciliary body (6), bovine ciliary body, and rat NPE and PE layers of the ciliary body and iris (35). In part, the apparently nonadditive effects of benzamil and dimethylamiloride might reflect biological variance. In addition, the same concentrations (10–20 \(\mu\)M) of amiloride, benzamil, and dimethylamiloride similarly inhibit the uptake of solute and water characterizing the regulatory volume increase of cultured human NPE cells (5). This is consistent with the pharmacological properties of low (L-type) amiloride-affinity Na\(^+\) channels (37) that also may be playing a role in the uptake of Na\(^+\) following incubation with ouabain. The data obtained

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**Fig. 7.** Posterior region protective effects of amiloride and its analogs in reducing ouabain-triggered changes in ionic composition of tissues untreated with heptanol. The results were obtained with anterior region sections from the same animals presented in Fig. 6, and the same treatments were followed. The normalized Na, K, and Cl contents are presented in A, B, and C, respectively. The normalized (Na\(^+\)+K\(^+\)+Cl\(^-\)) contents in D provide an index of HCO\(_3\)\(^-\) content. Control data were from 226 PE/NPE cell pairs from 28 sections. Data for ouabain treatment alone were from 196 PE/NPE cell pairs from 19 sections. Data for amiloride and ouabain treatment were from 134 PE/NPE cell pairs from 12 sections. Data for benzamil and ouabain treatment were from 112 PE/NPE cell pairs from 10 sections. Data for benzamil, DMA, and ouabain treatment were from 67 PE/NPE cell pairs from 8 sections. Data for DMA and ouabain treatment were from 65 PE/NPE cell pairs from 7 sections. *\(P < 0.05\) compared with cells exposed to ouabain alone (Ref). Significance was estimated by ANOVA, using the Bonferroni multiple comparisons test.
### Table 2. Effects of dimethylamiloride on posterior and anterior regions of ciliary epithelium

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<td>0.89 ± 0.12</td>
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<td>0.89 ± 0.14</td>
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<td>1.64 ± 0.20</td>
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<td>1.64 ± 0.36</td>
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<tr>
<td>Na⁺/K⁺ ratio</td>
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<td>P</td>
<td>0.010 &gt; 0.001</td>
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with ouabain suggest that Na⁺ can enter NPE cells through Na⁺ channels and likely Na⁺/H⁺ antiports far more rapidly in the anterior than in the posterior region of the ciliary epithelium.

It might seem that the results obtained by applying dimethylamiloride alone in the absence of ouabain (Table 2) are in conflict with the idea that Na⁺ entry via Na⁺/H⁺ exchange is important. In particular, one might expect that blocking Na⁺/H⁺ exchange, operating in isolation, would lead to reductions in intracellular Na⁺ content, contrary to observation. However, we have interpreted the regional difference in the K/Na ratio as an indication of a higher constitutive Na⁺ entry rate at the anterior NPE, and this difference is indeed reduced by dimethylamiloride. Under baseline conditions, the NPE cells of Table 2 displayed a ratio of 14.7 ± 1.3 posteriorly and 11.1 ± 0.8 anteriorly. As anticipated, dimethylamiloride reduced the regional difference in this ratio from 3.5 ± 1.6 under baseline conditions (P < 0.05) to 0.8 ± 1.1 (P > 0.4).

It is unclear why dimethylamiloride does not also lower the baseline Na⁺ contents. One speculation is that Na⁺/H⁺ exchange, in parallel with Cl⁻/HCO₃⁻ exchange, is very slow under baseline conditions. This speculation would leave unexplained our previous observations that either removal of CO₂/HCO₃⁻ or blockage of parallel Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchange with acetazolamide reduces baseline Cl⁻ content of the rabbit ciliary epithelial cells (31, 34). These maneuvers also reduce baseline net Cl⁻ flux across the isolated bovine ciliary epithelium (46), and acetazolamide inhibits aqueous humor formation by the perfused isolated bovine eye (43). An alternative explanation for dimethylamiloride not lowering the baseline Na⁺ contents is that dimethylamiloride's effects on ionic composition may be modified by the many transporters active in the plasma membrane, some of which are illustrated in Fig. 1. For example, compensation for dimethylamiloride-reduced entry of Na⁺ from the stroma into the PE cells could be provided by the Na⁺-K⁺-2Cl⁻ symport. Likewise, compensatory slowing of the turnover of the Na⁺,K⁺-activated ATPase (40) would tend to buffer the intracellular Na⁺ concentration; in the presence of ouabain, such buffering would be absent. Secondary effects possibly produced by dimethylamiloride on intracellular pH and Ca²⁺ concentration are expected to modify the activity of other transporters, particularly K⁺ channels. These compensatory and secondary actions may be of greater importance in stabilizing intracellular Na⁺ content under baseline conditions than during ouabain exposure, which might trigger enhanced Na⁺ entry.

Given the complex, incompletely understood interactions among these transport pathways, we suggest that the total Na⁺ + K⁺ content and the Cl⁻ content provide more robust indexes of the effects of blocking baseline Na⁺/H⁺ exchange than does Na⁺ content alone. Both of these indexes indicate that blocking Na⁺/H⁺ exchange in the absence of ouabain reduces cation and anion uptake anteriorly and posteriorly.

**Effects of hypotonic challenge.** Kregenow (28) first reported that hypotonicity triggered swelling of duck erythrocytes, followed by a second volume regulatory phase characterized by release of KCl and water. This secondary response, later termed the regulatory volume decrease (RVD), has been found to characterize nearly all cells (29), although the solute released varies with cell type. For example, K⁺ release can be
accompanied by HCO$_3^-$ and organic anions (29). We have observed a hypotonicity-triggered, progressive reduction in K/P in both NPE and PE cells that was threefold greater posteriorly than anteriorly. The reduction in cell K was associated with a fall in Cl/P and unmeasured anion content (Na + K − Cl)/(Na + K), and (Na + K − Cl)/(Na + K + Cl) (D) in tissues obtained from the same animals as in A and C. The data were obtained under isotonic conditions (0 min) and at 5, 20, and 40 min after incubation in solution containing 40 mM, rather than 110 mM, NaCl. The numbers of cells and sections analyzed at the different times (t) were as follows: isotonic: posterior, 93 PE/NPE cell pairs from 9 sections; anterior, 90 PE/NPE cell pairs from 6 sections; anterior, 67 PE/NPE cell pairs from 9 sections; t = 5 min: posterior, 61 PE/NPE cell pairs from 6 sections; anterior, 61 PE/NPE cell pairs from 7 sections; and t = 40 min: posterior, 101 PE/NPE cell pairs from 10 sections; anterior, 98 PE/NPE cell pairs from 11 sections. *P < 0.05; **P < 0.01; ***P < 0.001, significant difference between final and initial time points.

Since the RVD stimulates net Cl$^-$ secretion (14) and the RVD is much larger posteriorly than anteriorly (Fig. 8), we suggest that swelling activation of secretion is largely mediated in the posterior epithelium.

Summary. The present results further document the region dependence of ciliary epithelial transport. The baseline elemental contents and their response to hypotonic challenge suggest that the posterior epithelium is the major site of secretion. Consistent with this suggestion, the net thermodynamic driving force for solute uptake through the PE cell Na$^+$/K$^+$−2Cl$^-$ symport can be calculated from the current data to be threefold higher posteriorly than anteriorly. The effects of ouabain and amiloride and its analogs point to fine-tuning of aqueous humor formation by the anterior epithelium through substantial solute recycling at the aqueous surface and possibly through net transepithelial reabsorption. Selective stimulation of reabsorption by the anterior epithelium might provide a novel approach for reducing the rate of net aqueous humor formation, and thereby intraocular pressure.
REFERENCES


