Timolol may inhibit aqueous humor secretion by cAMP-independent action on ciliary epithelial cells

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McLaughlin, Charles W., David Peair, Robert D. Purves, David A. Carrè, Kim Peterson-Yantorno, Claire H. Mitchell, Anthony D. C. Macknight, and Mortimer M. Civan. Timolol may inhibit aqueous humor secretion by cAMP-independent action on ciliary epithelial cells. Am J Physiol Cell Physiol 281: C865–C875, 2001.—The β-adrenergic antagonist timolol reduces ciliary epithelial secretion in glaucomatous patients. Whether inhibition is mediated by reducing CAMP is unknown. Elemental composition of rabbit ciliary epithelium was studied by electron probe X-ray microanalysis. Volume of cultured bovine pigmented ciliary epithelial (PE) cells was measured by electronic cell sizing; Ca2+ activity and pH were monitored with fura 2 and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, respectively. Timolol (10 μM) produced similar K and Cl losses from ciliary epithelia in HCO3-/CO2 solution but had no effect in HCO3-/CO2-free solution or in HCO3-/CO2 solution containing the carbonic anhydrase inhibitor acetazolamide. Inhibition of Na+/H+ exchange by dimethylamiloride in HCO3-/CO2 solution reduced Cl and K comparably to timolol. CAMP did not reverse timolol’s effects. Timolol (100 nM, 10 μM) and levobunolol (10 μM) produced cAMP-independent inhibition of the regulatory volume increase (RVI) in PE cells and increased intracellular Ca2+ and pH. Increasing Ca2+ with ionomycin also blocked the RVI. The results document a previously unrecognized cAMP-independent transport effect of timolol. Inhibition of Cl-/HCO3 exchange may mediate timolol’s inhibition of aqueous humor formation.

electron probe X-ray microanalysis; cell volume; cell pH; cell calcium; chloride/bicarbonate exchanger; sodium/hydrogen exchanger

THE BILAYERED CILIARY EPITHELIUM secretes aqueous humor into the eye in three steps: stromal uptake by the pigmented ciliary epithelial (PE) cells, movement from the PE to the nonpigmented ciliary epithelial (NPE) cells, and release from the NPE cells into the posterior chamber of the eye (14, 16, 25, 37, 44, 63, 65, 66). The major solute components, Na+ and Cl−, are taken up from the stroma by the PE cell layer, diffuse through gap junctions (21, 25, 29, 50, 53, 56) to the NPE cells, and are then released into the aqueous humor. The first step, entry from the stroma, can proceed through paired NHE-1 Na+/H+ and AE2 Cl−/HCO3− antiports (22) and by a Na+-K+-2Cl− symport (23–25, 60). In the final step at the aqueous surface, Na+ is extruded by Na+-K+-ATPase and Cl− is released through Cl− channels of the NPE cells (36).

The intraocular pressure (IOP) reflects a balance between rates of formation and exit of aqueous humor. The IOP is usually elevated in glaucoma, a spectrum of blinding diseases treated with a wide range of agents, including carbonic anhydrase inhibitors, α-adrenergic and cholinergic agonists, prostaglandin analogs, and β-adrenergic blockers (55). The nonselective β-adrenergic blocker timolol (73) is among the most widely used and effective drugs in lowering the secretory rate, and thereby the IOP (28). Timolol binds to β-adrenergic receptors of the ciliary processes with high affinity (62). Agonists to all β-adrenergic receptors (β1, β2, and β3) stimulate adenyl cyclase via interaction with Gs to increase cAMP production (34). As a known β-blocker, timolol could act by reducing the intracellular concentrations of cAMP. However, it has long been unclear whether the putative reduction in cAMP itself causes the reduction in IOP because of the following considerations (69). First, a surprisingly high concentration of timolol is considered necessary to lower IOP. The ocular hypertensive rabbit (following water load or α-chymotrypsin injection) has been used to mimic the glaucomatous state. In that case, 0.5% topical timolol has been required to reduce the IOP, a concentration 500 times larger than that (0.0001%) needed to inhibit the hypotensive effect of the β-adrenergic agonist isoproterenol in the same study (62). An ocular hypotensive effect has also been reported in normotensive rabbits at very low timolol concentration [0.01% topical timolol (31)], but the effect is small. Second, β1-adrenergic antagonists are effective in some models of ocular hypertension (6, 61), although the high density of β-receptors in the ciliary process are predominantly of the β2 subtype. Third, d-timolol may be as effective as

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l-timolol (42) in decreasing aqueous flow, despite steer-
|specificity of the β-adrenergic receptors for the i-iso-
|mers (41, 49). Fourth, if timolol reduces aqueous
|humor formation by blocking β-adrenergic-mediated
|increase of cAMP production, we would expect cAMP
|itself to increase inflow. However, cAMP certainly does
|not markedly increase aqueous flow, and some inves-
tigators have observed a decrease in inflow following
|administration of forskolin to stimulate endogenous
|production of cAMP (11, 40). Fifth, one might ascribe
|the absence of a marked increase in inflow following
|cAMP administration to chronic in vivo β-adrenergic
|stimulation, blunting the effects of exogenous cAMP.
|However, such constant agonist stimulation would de-
sensitize the β-adrenergic receptors, uncoupling the
|adenyl cyclase system (5, 48). The foregoing consid-
erations do not preclude the possibility that timolol
|reduces secretion of aqueous humor exclusively
|through its action as a nonselective β-adrenergic an-
tagonist, but have raised doubts about that hypothesis.

We have addressed the issue by electron-probe X-ray
microanalysis of the ciliary epithelial cells in the intact
rabbit ciliary epithelium (7, 44). The great advantage
of this method is the unique capability of quantifying
the Na, K, and Cl contents at visualized sites within
individual cells (e.g., chapter 6 of Ref. 18). Because of
the complexity of the ciliary epithelium, the electron
microprobe analyses have been complemented with
fluorometric and volumetric measurements of cultured
bovine PE cells that we have already characterized (22,
47). The results of the present study suggest an alter-
native mechanism by which timolol could reduce aque-
ous inflow and thus IOP.

MATERIALS AND METHODS

Methods used in this study have been described in detail
elsewhere (7, 22, 44, 47).

Cellular model: rabbit ciliary epithelium. Dutch-belted
rabbits of either sex and older than 6 wk postweaning were
obtained from the Department of Laboratory Animal Sci-
ences, University of Otago Medical School, Dunedin, New
Zealand, and were treated in accordance with the Associa-
tion for Research in Vision and Ophthalmology statement for
the Use of Animals in Research. The animals were anesthetized
with 30 mg/kg pentobarbital sodium and killed by injecting
air into the marginal ear vein. After enucleation, the iris-
ciliary body was excised, cut into quarters, and each quarter
perfused with a solution similar to that previously described
(47) (in mM): 114 Na^+ , 2.5 Ca^{2+}, 1.2 H_2PO_4, 25–30 HCO_3^-,
and 10 glucose at pH 7.30–7.45 and 305–315 mosmol/kgH_2O.
-HCO_3^-free solution was prepared by isosmolar replacement
of HCO_3^- with Cl^- . Hypotonic HCO_3^-containing solution
(150–160 mosmol/kgH_2O) was prepared by reducing the
NaCl concentration to 79.5 mM. Depending on whether or
not HCO_3^- was included, the gas bubbled through the solu-
tion throughout incubation of the iris-ciliary bodies consisted
of either 95 %O_2-5 %CO_2 or pure O_2 respectively. Cl^-free
solutions were prepared by replacing MgCl_2 with MgSO_4
and by replacing the remaining Cl^- with methysulfonate. In
conducting fluorescence measurements, bovine PE cells were
perfused with a solution similar to that previously described
(47) (in mM): 114 Na^+, 5.9 K^+, 122.1 Cl^-, 15.0 HEPES, 1.2 Mg^{2+},
1.2 H_2PO_4, 25–30 HCO_3^- , and 10 glucose at pH 7.30–7.45 and 305–315 mosmol/kgH_2O.
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All chemicals were reagent grade. Bovine albumin (RIA
grade; Immuno Chemical Products) was dialyzed for 48–60
h, freeze-dried at –70°C, and stored at 4°C. A 30% (wt/vol)
solution was prepared by dissolving the albumin in the same
medium in which the tissue was incubated. Timolol maleate
was added to the incubation media from stock solutions
in water or ethanol, and dimethylamiloride from stock solutions
in ethanol. Dimethylamiloride (dibutyryl cAMP) and 8-bromoadenosine
3’-5’-cyclic monophosphate (dibutyryl cAMP) and 8-bromoadenosine 3’-5’-cyclic mono-
phosphates were dissolved directly in incubation media. Acet-
azolamide was added from stock solutions in dimethylform-
amide. In all cases, the same concentration of solvent vehicle
(0.1% vol/vol) was applied to parallel control preparations. Timolol and propranolol were obtained from Sigma, and
levobunolol was purchased as Betagan (Allergan, Hor-
migueros, Puerto Rico).

Electron microprobe data acquisition and reduction. After
incubation, the tissues were blotted and a 30% albumin
solution was applied briefly to the epithelial surface of the
NPE cells (i.e., to the basement membrane supporting the
NPE cells). Excess albumin was removed by blotting, and the tissue segment was then plunged into liquid propane at −180°C to freeze the preparation quickly before ions and water could undergo redistribution. Sections were then cut 0.2–0.4 μm in thickness at −80°C to −90°C with a cryoultramicrotome, 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. Unless otherwise stated, five to eight pairs of NPE and PE cells were measured in each of two sections cut from each quadrant.

Electron probe X-ray microanalysis permits both quantification and localization of intracellular elements. Using an electron microscope, we target a specific visualized area within the cell. The specimen is irradiated with a beam of electrons, which ionizes a small fraction of the atoms bombarded. After an electron is knocked from an inner atomic shell, an electron from an outer shell can take its place. The relaxation of the electron from a higher to a lower energy state generates a quantum of X-ray energy. Spectroscopic measurement of the characteristic energy and number of these quanta permits identification and quantification of the elements within the sample.

The dried sections were imaged with a transmitted electron detector. Measurements were collected with a Tracor Northern X-ray 30-mm2 detector, using a probe current of 100 s 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.

The elemental peaks were quantified by filtered least-square fitting to a library of monoelemental peaks (8). 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 nonnucleus contributions arising from the Al specimen holder and Ni grid.

As previously discussed (44), for purposes of data reduction the elemental peaks were routinely normalized to the phosphorus signal obtained in the same scanned area of each cell. 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 (Pi) is accumulated to only 3 mmol/kg intracellular water in the epithelial cells of frog skin (20). In such cells, the total pool of ATP, ADP, phosphocreatine, and P, corresponds to only 5% of the total P pool (~400–500 mmol/kg dry wt) measured in the ciliary epithelial cells (7). The validity of normalizing to P has been experimentally confirmed by the close linear relationship between the contents of the two largely intracellular elements, K and P (see Fig. 3 of Ref. 7).

The values we report for Na/P, Cl/P, and K/P are the measured normalized estimates of the intracellular Na, Cl, and K contents, respectively. Because the NPE and PE cells responded similarly to the experimental changes (Table 1), the data from the two cell types have generally been pooled. Although it is not possible to calculate ion concentrations in millimoles per liter from these data, the intracellular contents of (Na + K) or of (Na + K + Cl) provide indexes of intracellular water content (1). For this reason, the measured values of (Na + K)/P and of (Na + K + Cl)/P are entered in the Table and cited in the text, where appropriate. The parameter (Na + K)/P is the normalized difference between the principle mobile cations and anion and provides an index of the tissue content of unmeasured anion; this index is also presented in the Table.

Values are presented as means ± SE. The numbers of cells analyzed are indicated by the symbol n, while N is used to refer to numbers of experiments. With more than two groups of electron microprobe data, the 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 posttest. With two groups, the nonparametric Mann-Whitney test was used.

In principle, intracellular Na+, K+, and Cl− concentrations might also be measured simultaneously fluorometrically. Membrane-permeant probes are available for this purpose. However, differential bleaching and leakage of the fluorophores, limited selectivity of the K+ probe, and the effects of quenching by uncontrolled factors constitute significant challenges. For example, intracellular fluorescence of the Cl−-sensitive fluorophore 6-methoxy-N-(3-sulfopropyl)-quinolinium has been used to measure intracellular volume, based on quenching by unidentified intracellular anions and proteins (58). It is entirely feasible to measure intracellular pH (pH7) and Ca2+ activity simultaneously using ratiometric dyes (described in Fluorescence experiments). Because the excitation

Table 1. Effects of timolol in HCO3−/CO2 solutions: all available results

<table>
<thead>
<tr>
<th></th>
<th>NPE and PE cell types combined</th>
<th>NPE cells</th>
<th>PE cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Na</td>
<td>Cl</td>
<td>K</td>
</tr>
<tr>
<td>Controls</td>
<td>346</td>
<td>0.097±0.005</td>
<td>0.312±0.005</td>
</tr>
<tr>
<td>+Timolol</td>
<td>570</td>
<td>0.092±0.002</td>
<td>0.253±0.003</td>
</tr>
<tr>
<td>Differences</td>
<td>-0.005±0.004</td>
<td>-0.059±0.006</td>
<td>-0.064±0.010</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
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</table>

Values are means ± SE and are from 80 sections of 16 animals; n = no. of cells. NPE, nonpigmented ciliary epithelial; PE, pigmented ciliary epithelial; NS, not significant.
or emission spectra are different for the free and bound forms of such fluorophores, the ratio of measurements at two different frequencies permits measurement of ion activity independent of photobleaching and total dye concentration. In the absence of satisfactory ratiometric dyes for measuring intracellular Na, K, and Cl simultaneously, we conclude that electron microprobe X-ray analysis provides a unique opportunity for this purpose in preparations displaying cellular heterogeneity.

**Volumetric measurements and analysis.** After harvesting cells from a T-75 flask by trypsinization (19), a 0.5-ml aliquot of the cell suspension in DMEM was added to 20 ml of each test solution. Parallel aliquots of cells were studied on the same day. One or two aliquots served as control, and the others were exposed to different experimental conditions at the time of suspension. The same amount of solvent vehicle was always added to the control and experimental aliquots. The sequence of studying the suspensions was varied to preclude systematic time-dependent artifacts. Cell volumes of isosmotic suspensions were measured with a Coulter counter (model ZBI-Channelyzer II), using a 100-μM isosmotic solution. Cell volumes was always added to the control and experimental aliquots. The time of suspension. The same amount of solvent vehicle was always added to the control and experimental aliquots.

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**Fluorescence experiments.** For measurements of intracellular Ca²⁺, cells grown on coverslips for 1–5 days were loaded in the dark with 5 μM fura 2-AM and 0.005% Pluronic F-127 (Molecular Probes, Eugene, OR) for 50–240 min at 25°C or 37°C, then rinsed and maintained in fura-free solution at 1 Hz following excitation at 484 and 440 nm, and the ratio was determined with a Delta-Ram system and Felix software. pH calibration, based on that of Wu et al. (67), was performed by perfusing the cells with 110 mM KCl, 20 mM NaCl, 20 μM nigericin, and 20 mM buffer (pH 7.0 solution buffered with MES, pH 7.4 with PIPES, pH 7.4 with HEPES, and pH 8.0 with TES). In experiments measuring Ca²⁺ and pH simultaneously, both dyes were loaded together, and the excitation wavelength alternated among 340, 380, 484, and 440 nm with 1-s exposure to each wavelength. Calibration to absolute pH or Ca²⁺ values was usually not performed when both dyes were present; the appropriate ratios provided indexes of Ca²⁺ and pH.

**RESULTS**

**Effects of timolol on epithelial cell composition in tissues incubated in the presence or absence of HCO₃⁻/CO₂ solution.** Timolol was applied at a concentration of 10 μM, within the concentration range likely reached clinically in the aqueous humor. Instillation of 20–50 μl timolol (0.5%) into the rabbit conjunctival sac can be calculated to produce peak concentrations of ~8 μM (62) to 17 μM (51). As considered in the Discussion, Vareilles et al. (62) found that a timolol concentration of 8 μM was required to reduce the IOP of rabbits after water loading. The same concentration we have chosen has also been used in other in vitro studies of timolol’s mode of action (39).

In the nominal absence of HCO₃⁻/CO₂, timolol produced no significant changes in epithelial cell Na, Cl, or K (Fig. 1). In contrast, in ciliary tissue from the same eyes incubated in HCO₃⁻/CO₂, 10 μM timolol produced significant losses of Cl and K (Fig. 1). A time course was obtained in a separate experiment conducted with HCO₃⁻/CO₂ solution. Significant losses of Cl (P < 0.001) and K (P < 0.05) were detected by 10 min. Altogether, in our studies we have obtained data from 32 eyes in which tissues were incubated in HCO₃⁻ with or without timolol for 20–30 min (Table 1). Averaging the results obtained with equal numbers of NPE and PE cells (Table 1), there were highly significant (P < 0.001), comparable losses of Cl (ΔCl/P) = −0.059 ± 0.006) and K (ΔK/P) = −0.064 ± 0.010) content, accompanied by significant water loss (indicated by the reductions in both ([Na + K]/P) and ([Na + K + Cl]/P)). In contrast, there was no significant fall in unmeasured anion content, monitored by [Na + K – Cl]/P). Constancy of unmeasured anion content with a fall in intracellular water suggests that the intracellular HCO₃⁻ concentration rose as the tissues lost Cl. The same conclusions were reached by considering the NPE and the PE cells separately (Table 1). As previously noted and discussed (44), the phosphorus-normalized
bicarbonate-free solution
bicarbonate solution

Fig. 1. Effects of timolol (10 µM) on ciliary epithelial Na/P, Cl/P, or K/P ratios in HCO$_3$-free or HCO$_3$ solutions. In these box plots, 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. The open and shaded symbols present control and experimental results, respectively. Significant differences from controls: *P < 0.05, **P < 0.01, ***P < 0.001. Data were obtained from experiments using eyes from 2 animals as follows: for controls, 8 sections were analyzed, with 6 nonpigmented ciliary epithelial (NPE) and 6 pigmented ciliary epithelial (PE) cells measured in each section, giving a total of 96 cell measurements for each condition.

Contents of Na, K, and Cl are higher in NPE than in PE cells. Neverthess, the timolol-triggered reductions of Cl were similar in the NPE (~20%) and PE (~17%) cells, reflecting the syncytial nature of the ciliary epithelium under baseline conditions.

Effects of cAMP on epithelial composition in tissues incubated in HCO$_3$-CO$_2$ solution. As a known β-blocker, timolol might act by reducing cell cAMP levels (Introduction). If so, this blocking action should be circumvented by directly adding a membrane-permeant form of cAMP (dibutyryl cAMP). We have previously observed that the specific choice of membrane-permeant form is not significant (13, 19, 27). Dibutyryl cAMP (1 mM) did have an effect on ion transport by the ciliary epithelium, reducing cell K significantly (Fig. 2). However, the cAMP did not alter cell Cl (Fig. 2). Furthermore, the cyclic nucleotide did not reverse the effects of 10 µM timolol in reducing both Cl and K when both agents were added simultaneously (Fig. 2). Instead, the combination of the two agents appeared to be additive, with the Cl loss slightly greater than for timolol alone, and a loss of K that was twice as great as the loss of Cl.

Effects of acetazolamide and timolol on epithelial composition in tissues incubated in HCO$_3$-CO$_2$ solution. In previous studies, we showed that the carbonic anhydrase inhibitor acetazolamide decreases cell Cl and K (7, 44). We ascribed these effects to inhibition of the rate of cellular production of H$^+$ and HCO$_3^-$, reducing the rates of Na$^+$/H$^+$ and Cl$^-$/HCO$_3^-$ exchange. In turn, the decreased rate of Na$^+$ entry reduced the rate of Na$^+$ extrusion and K$^+$ uptake by the Na$^+$/K$^+$-ATPase. The overall effect was a decrease in cell Cl and K, with relatively little change in cell Na. Because timolol also decreased cell Cl and K, we compared the effects of acetazolamide and timolol (Fig. 3). In this set of experiments, Cl/P was decreased by 0.5 mM acetazolamide (~0.123 ± 0.012) to a greater extent than it was by 10 µM timolol (~0.045 ± 0.013). However, the two effects were not additive, since the combination of inhibitors caused no greater reduction in Cl/P (~0.104 ± 0.021) than did acetazolamide alone.

The nonadditivity of timolol and acetazolamide was surprising in view of clinical experience. However, Berson and Epstein (4) have pointed out that the inflow inhibitions of the two drugs are less than additive. Direct addition of the drugs to the well-stirred extracellular fluid may permit more complete inhibition of the transport processes in vitro, precluding additivity of the two effects.

Effects of dimethylamiloride on epithelial composition in tissues incubated in HCO$_3$-CO$_2$ solution. Because it was possible that timolol was affecting some
Levobunolol, another nonselective β-adrenergic receptor blocker widely used in treating glaucoma, mimicked the effects of timolol in blocking the RVI at the same 10 μM concentration (Fig. 5C). Interestingly, a third nonselective β-blocker (propranolol), which is not commonly used for lowering IOP, was found to have no effect on the RVI at either 100 nM (N = 3) or 10 μM (N = 3, data not shown).

**Effects of timolol on pH**. The preceding results suggest that timolol might reduce ciliary epithelial secretion by inhibiting either the Na+/H+ or Cl−/HCO3− antiport, thereby interfering with the paired uptake of Na+ and Cl− in exchange for H+ and HCO3−. To determine which of the two paired exchangers might be the primary target of timolol’s action, we monitored changes in the pHi of the bovine PE cells directly with the fluorescent pH indicator BCECF. The baseline value was 7.38 ± 0.02 (N = 14). Timolol clearly and repeatedly raised pHi, and the elevation was frequently reversible on removal of timolol. Calibration showed that 10 μM timolol elevated pHi by 0.027 ± 0.007 units (N = 7, P < 0.01). The probability of elevation was concentration dependent, with 10 μM timolol raising pH in 83% of trials, whereas 100 nM produced an alkalinization only 25% of the time.

**Effects of timolol on intracellular Ca2+**. Timolol’s actions on ion concentration and cell volume appeared unrelated to the drug’s inhibition of cAMP production. However, complex interactions have been noted among timolol, cAMP, and Ca2+ (33, 45, 52, 59, 70–72), so we looked at Na+/H+ and Cl−/HCO3− exchange, we studied the effects of a known inhibitor of Na+/H+ exchange, dimethylamiloride (Fig. 4). Its effects at 50 μM were similar to those of 10 μM timolol, with significant reductions in cell Cl and K.

**Effects of timolol, levobunolol, and propranolol on RVI of PE cells**. If timolol acts at a single site both to inhibit aqueous humor secretion and reduce epithelial cell Cl content, that site must be the PE cells at the stromal surface (see discussion). To test this hypothesis more directly, we monitored the RVI as an index of solute and water uptake by the PE cells (see materials and methods). Application of 50% hypotonicity followed by restoration of isotonicity produced an RVI in control preparations (Fig. 5, A and C, and Fig. 6, A–C). Timolol blocked the RVI at 10 μM concentration (Fig. 5A). Incomplete inhibition was also observed at a 100-fold lower concentration (100 nM) in the experiments of Fig. 6A. As in the case of the timolol-triggered reduction in epithelial Cl content, cAMP did not protect against the timolol-triggered inhibition (Fig. 6B). The effect of timolol was dependent on extracellular Cl concentration. In the absence of external Cl−, the cells displayed neither an RVI nor a response to timolol (Fig. 5B).
Simultaneous effects of timolol on pH and Ca\textsuperscript{2+}. Given the ability of timolol to elevate both pH and Ca\textsuperscript{2+}, experiments were designed to measure both parameters simultaneously in an attempt to determine any temporal linkage of the two effects. As indicated by Fig. 7B, cells loaded with both fura 2 and BCECF showed that Ca\textsuperscript{2+} and pH usually rose together. Positive correlation, defined as a change, or lack thereof, in both Ca\textsuperscript{2+} and pH was seen in 17/22 experiments, indicating that the effects on the two parameters were linked. It should be noted that the lack of perfect correlation supports the independence of either measurement. Although calibration to absolute pH or Ca\textsuperscript{2+} levels was not always performed when both dyes were present, the ratio of light excited at 340 nm to that at 380 nm was used as an index of changes in free Ca\textsuperscript{2+} concentration, and the ratio of light excited at 480 nm to that at 440 nm was used to monitor changes in pH. The ratio for Ca\textsuperscript{2+} was elevated by 0.012 by 1 nM also measured Ca\textsuperscript{2+} directly with the fluorescent indicator fura 2. Timolol clearly and repeatedly raised intracellular Ca\textsuperscript{2+}. Levels rose steadily, showing little or no reduction in the continued presence of timolol for as long as 5 min (Fig. 7A). Levels of intracellular Ca\textsuperscript{2+} generally returned to baseline once the timolol was washed off. The success rate was concentration dependent, with 10 \mu M timolol elevating intracellular Ca\textsuperscript{2+} in 83\% of attempts and 100 nM timolol elevating Ca\textsuperscript{2+} only 54\% of the time. In a portion of the experiments the absolute levels of intracellular Ca\textsuperscript{2+} were calibrated from the ratio of light excited at 340 nm to that at 380 nm. At 10 \mu M, timolol increased intracellular Ca\textsuperscript{2+} by 57 \pm 20 nM from a baseline of 77 \pm 7 nM, raising intracellular Ca\textsuperscript{2+} levels to between 91 and 190 nM (N = 14, P < 0.05). The magnitude of the response was dependent on concentration, as 100 nM raised intracellular Ca\textsuperscript{2+} by 13 \pm 4 nM (N = 4) and 1 nM produced only a 4 \pm 1 nM rise (N = 4).

Fig. 5. Effects of timolol and levobunolol on cell volume of bovine PE cells. A: timolol (10 \mu M) blocked the regulatory volume increase of bovine PE cells. Data points are means of 10 control and experimental aliquots and have been normalized to their volumes (v\textsubscript{c}, in \%) at t = 28 min. The control trajectories from cells suspended in Cl\textsuperscript{-} containing solution in Figs. 5 and 6 have been fit to the monoexponential: v\textsubscript{c} = a \exp(t/\tau). In A, a = 2.2 \pm 0.4\% and \tau = 13.5 \pm 4.2 min. In Fig. 5, A and C, and Fig. 6, A–C, the sets of experimental and control data points are significantly different (P < 0.01 by the F distribution). B: in the absence of extracellular Cl\textsuperscript{-}, the regulatory volume increase (RVI) was abolished and timolol exerted no effect on cell volume (N = 5). C: levobunolol (10 \mu M) mimicked the effects of 10 \mu M timolol, blocking the RVI. Fit of the control data generated values of a = 3.5 \pm 0.3\% and \tau = 7.7 \pm 1.9 min (N = 6).

Fig. 6. Dependence of timolol effect on concentration and interactions with cAMP and Ca\textsuperscript{2+}. A: timolol partially inhibited the RVI of bovine PE cells at 100 nM. The control data have been obtained from 12 aliquots and fit with a = 9.3 \pm 0.4\% and \tau = 19.6 \pm 1.2 min. The means at timolol concentrations of 100 nM and 10 \mu M were obtained from 6 aliquots each. B: cAMP (500 \mu M) did not prevent the inhibition of RVI triggered by exposing bovine PE cells to 10 \mu M timolol. Duplicate controls (N = 10) were studied in parallel with the timolol (N = 5) and cAMP + timolol (N = 5) experimental aliquots. The control data were fit with a = 7.1 \pm 0.7\% and \tau = 17.2 \pm 2.7 min. C: the calcium ionophore ionomycin (2 \mu M, N = 4) blocked the RVI displayed by aliquots (N = 6) of control bovine PE cells. The control data were fit with a = 8.4 \pm 1.3\% and \tau = 22.5 \pm 5.2 min.
timolol \((N = 1)\), \(0.021 \pm 0.006\) by 100 nM \((N = 6)\), and \(0.039 \pm 0.009\) by 10 \(\mu\)M \((N = 14)\). The ratio for \(pH\) was elevated by \(0.015\) \((N = 2)\) by 1 nM timolol, \(0.032 \pm 0.012\) by 100 nM \((N = 5)\), and \(0.067 \pm 0.02\) by 10 \(\mu\)M \((N = 12)\). These data establish that timolol raises both the intracellular Ca\(^{2+}\) and \(pH\) of PE cells.

**Effects of levobunolol on \(pH\) and Ca\(^{2+}\).** The \(\beta\)-blocker levobunolol also elevated intracellular Ca\(^{2+}\) and \(pH\) levels, although the effects were smaller. At 100 nM, levobunolol had no detectable effect on either parameter, but \(pH_i\) was elevated \(0.012 \pm 0.003\) pH units by 10 \(\mu\)M levobunolol \((N = 8, P < 0.01)\), while the ratio of light excited by irradiating fura 2 at 340/380 rose \(0.016 \pm 0.003\) \((N = 13, P < 0.001)\).

**Effects of ionomycin on RVI of PE cells.** Because timolol both increased intracellular Ca\(^{2+}\) concentration and inhibited the RVI, we wondered whether increasing Ca\(^{2+}\) concentration by another means would also inhibit RVI. Application of the calcium ionophore ionomycin (2 \(\mu\)M) indeed blocked the RVI (Fig. 6C).

**DISCUSSION**

The central observations of the present study are that both timolol and a membrane-permeant form of cAMP exert effects on the intracellular electrolyte composition of the NPE and PE ciliary epithelial cells, but these effects are not opposite to one another. Adding cAMP not only did not reverse the effects of timolol, but produced additive actions. Likewise, cAMP did not reverse the inhibition of the RVI produced by applying timolol to isolated bovine PE cells. Thus neither the reduction in epithelial Cl content nor the inhibition of solute uptake by PE cells produced by timolol can be ascribed to a reduction in cAMP production.

Dibutryl cAMP triggered cell K loss in a HCO\(_3^-\)/CO\(_2^-\) bath, but a reduction of cell Cl was not detected (Fig. 3). The K loss may reflect activation of basolateral K\(^+\) channels, as seen in other secretory epithelia (15, 30). Because macroscopic electroneutrality must be preserved, this cAMP-triggered K loss may have been accompanied by a net loss of HCO\(_3^-\), as found in other epithelial cells (3, 10, 57). It is known that cAMP exerts multiple effects specifically on PE cells, including direct activation of Cl\(^-\) channels leading to Cl\(^-\) release (27) and stimulation of Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport through activation of protein kinase A (54), which can lead to either uptake or release of Cl\(^-\) depending on the ambient thermodynamic driving forces (44). Thus the
net change in Cl content produced by cAMP depends on a complex interaction of multiple effects, possibly accounting for certain apparently paradoxical results in the literature. For example, β-adrenergic agonists such as isoproterenol, which stimulate cAMP production, have been reported to increase aqueous humor inflow (9). In contrast, forskolin, which also stimulates cAMP formation, has been found to reduce inflow (11, 40), and isoproterenol itself has also been reported to reduce IOP in water-loaded rabbits (62). Whatever the nature of the complex interactions, the effects of timolol and cAMP on the intact ciliary epithelium were additive in the present study. This finding demonstrates that timolol can act independently of cAMP-mediated pathways.

Site of action. Timolol inhibits secretion of aqueous humor, the major anionic component of which is Cl−. Timolol could inhibit secretion by blocking Cl− uptake by the PE cells at the stromal surface, thereby reducing intraepithelial Cl content. Alternatively, timolol could reduce Cl− release by the NPE cells at the aqueous surface, thereby increasing Cl content. The microanalyses of intact rabbit ciliary epithelium demonstrated a fall in intracellular Cl content, strongly suggesting that the dominant action of timolol is at the stromal surface. This deduction was tested by monitoring fluid uptake during the course of the RVI by isolated cultured bovine PE cells. The RVI reflects cellular uptake of ions to replace those lost during hypotonic exposure. In these bovine cells, RVI depends on solution HCO3−/CO2 and Na+/H+ exchange activity (22), supporting the idea that normally much of the NaCl uptake step at the stromal surface is mediated by paired Cl−/HCO3− and Na+/H+ antiports (37, 44, 65). Timolol blocked the RVI at the same concentration (10 μM) used in the microprobe studies and even exerted a partial block at a 100-fold lower concentration (100 nM). In the absence of Cl− in the bath, the RVI was abolished and timolol had no further effect. These data support the idea that timolol acts at the stromal side to block NaCl uptake by the paired antiports, recently identified to be NHE-1 Na+/H+ and AE2 Cl−/HCO3− exchangers (22). Further support is provided by the observations that reducing delivery of HCO3− and H+ to the paired antiports (by inhibiting carbonic anhydrase) and directly inhibiting the Na+/H+ antiport (with dimethylamiloride) mimicked the changes in intracellular composition caused by timolol.

In principle, the roles of the Cl−/HCO3− and Na+/H+ antiports could be very different in the rabbit tissue that we studied by microprobe analysis and the bovine preparation that we studied volumetrically and fluorometrically. However, recent reports indicate that these antiports likely play an important role in NaCl uptake from the stroma by PE cells from both species (22, 44, 60). The agreement of the results obtained with tissues from the two species and using different experimental techniques further supports the conclusion that timolol likely inhibits stromal uptake of NaCl by PE cells.

We have tested the effects of two additional nonspecific β-blockers (levobunolol and propranolol) on the RVI of bovine PE cells. At 10 μM concentration, levobunolol mimicked the inhibitory effect of the same concentration of timolol. In contrast, 10 μM propranolol was ineffective. These observations conform to clinical experience. Both levobunolol and timolol have been widely used in the topical treatment of glaucoma, but propranolol has not. It should be noted that propranolol and timolol have been reported to have a number of very different effects on different cell preparations. For example, 32P incorporation into phosphatidylinositol 4,5-bisphosphate of cat iris and ciliary process is increased by propranolol but is decreased by timolol (70). In part, the differences reported may reflect the sevenfold higher potency of timolol over propranolol for β-adrenergic receptors in the rabbit iris-ciliary body (2). However, given the different structures of the β-blockers, it is also possible that actions in addition to binding to β-adrenergic receptors may well be involved.

Transport protein target. To test whether the dominant inhibitory effect of timolol is on Cl−/HCO3− or Na+/H+ antiports, we monitored pH of the bovine PE cells. Timolol (and to a lesser extent, levobunolol) increased pH, suggesting that the primary effect is inhibition of Cl−/HCO3− exchange, thereby slowing extrusion of base out of the cell.

Second messenger. The current data argue against the possibility that timolol exerts its transport effects solely by inhibiting cAMP formation. Given the interactions reported for timolol, cAMP, and Ca2+ (33, 45, 52, 59, 71, 72), we also monitored intracellular Ca2+ activity. Timolol increased Ca2+ activity over a broad range of concentrations (1 nM to 10 μM). We do not know whether the elevated Ca2+ activity triggers the putative inhibition of Cl−/HCO3− exchange, but the near synchrony of changes in Ca2+ and pH and the block of the RVI by ionomycin do raise this possibility. This suggestion is consistent with the report of Tachado et al. (59), who observed that the β-adrenergic agonist isoproterenol produced muscle relaxation at an order of magnitude lower concentration than that required to accumulate cAMP or inhibit inositol 1,4,5-trisphosphate formation in the bovine iris sphincter. The two studies raise the possibility that the agonist [isoproterenol (59)] and the antagonist (timolol at clinically relevant concentrations in the present work) act on β-adrenergic receptors that exert effects independent of changes in intracellular cAMP concentration. Whether or not Ca2+ indeed inhibits the anion exchanger directly and the mechanism by which timolol elevates intracellular Ca2+ activity remain to be determined.

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