cAMP-activated maxi-Cl\textsuperscript{−} channels in native bovine pigmented ciliary epithelial cells

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Do, Chi-Wai, Kim Peterson-Yantorno, Claire H. Mitchell, and Mortimer M. Civan. cAMP-activated maxi-Cl\textsuperscript{−} channels in native bovine pigmented ciliary epithelial cells. Am J Physiol Cell Physiol 287: C1003–C1011, 2004. First published June 9, 2004; 10.1152/ajpcell.00175.2004.—The eye’s aqueous humor is secreted by a number of species including rabbit, monkey, and human (7, 8, 25, 32). Cl\textsuperscript{−} can then diffuse through the intercellular gap junctions to the NPE cells and finally exit through Cl\textsuperscript{−} channels to the posterior chamber of the eye (11, 18, 54, 55). Many transport mechanisms underlying aqueous humor secretion have been identified, but the regulatory pathways remain elusive.

cAMP has long been proposed to play an important role in modulating the rate of aqueous humor formation and IOP but by mechanisms and regulatory pathways that are yet unclear (reviewed recently by Do and Civan, Ref. 17). Administration of isoproterenol and forskolin, which trigger cAMP production, reduces aqueous humor formation and IOP among different species including rabbit, monkey, and human (7, 8, 25, 32). The addition of terbutaline, another \beta\textsuperscript{-}adrenergic agonist, to the arterially perfused bovine eye reduces the rate of aqueous humor formation (46). Recently, cAMP has been found to activate whole cell Cl\textsuperscript{−} currents of immortalized bovine PE cells (24). Indirect evidence also suggested that cAMP might trigger activation by acting directly on Cl\textsuperscript{−} channels, independent of cAMP-activated kinase (PKA). Large-conductance maxi-Cl\textsuperscript{−} channels have not been noted in PE cells (38), but their role in ciliary epithelial secretion is unknown. As in other cells, the physiological significance of these channels is unclear, because their open-channel probability (P\textsubscript{o}) is maximum within ±20 mV (6), far from the ciliary epithelial membrane potential (V\textsubscript{m}) of approximately −60 mV (9, 26).

In the present study, we examine the effects of cAMP on excised single-channel and whole cell currents and on cell volume in native bovine PE cells. Our results suggest that cAMP activates maxi-Cl\textsuperscript{−} channels in PE cells, facilitating Cl\textsuperscript{−} release from the PE cells to the ciliary stroma. This Cl\textsuperscript{−} recycling process may potentially serve as a pathway to regulate net aqueous humor formation.

MATERIALS AND METHODS

Isolation of fresh native bovine PE cells. Freshly nucleated bovine eye were collected from a local abattoir. The cornea and iris were removed along the limbs. After that, ciliary processes were excised into small pieces and rinsed with Dulbecco’s phosphate-buffered saline (PBS). The retina was then peeled off the ciliary processes, and the ciliary epithelium was isolated. The ciliary epithelium was then cut into small pieces and washed with PBS. The ciliary epithelium was then transferred to a small dish containing PBS.

Active ion transport across the ciliary epithelium, followed by osmotic water movement (30). Cl\textsuperscript{−} is the principal anion of the aqueous humor, and transepithelial Cl\textsuperscript{−} secretion likely plays a central role in aqueous humor formation (12, 14, 18, 51). Figure 1 illustrates a consensus model of aqueous humor formation. Cl\textsuperscript{−} can enter from the ciliary stroma into the PE cells through two main transcellular pathways: the Na\textsuperscript{+}–K\textsuperscript{+}–2Cl\textsuperscript{−} cotransporter and parallel Na\textsuperscript{+}/H\textsuperscript{+} and Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchangers (14, 18, 20, 35, 52). Cl\textsuperscript{−} can then diffuse through the intercellular gap junctions to the NPE cells and finally exit through Cl\textsuperscript{−} channels to the posterior chamber of the eye (11, 18, 54, 55). Many transport mechanisms underlying aqueous humor secretion have been identified, but the regulatory pathways remain elusive.

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GLAUCOMA IS A GROUP OF CONDITIONS in which retinal ganglion cells are lost, at least in part, because of events triggered by an increase in intraocular pressure (IOP). The level of IOP is governed by the dynamic balance between aqueous humor formation and drainage. Although glaucoma is believed to be due to an increase in outflow resistance, a major strategy for reducing IOP is to decrease the rate of aqueous humor formation. Aqueous humor is secreted by the ciliary epithelium, which comprises two epithelial layers: an outer pigmented ciliary epithelial (PE) layer adjacent to the ciliary stroma, and an inner nonpigmented ciliary epithelial (NPE) layer abutting the aqueous humor. Cells within and between these two layers are functionally coupled through intercellular gap junctions (41). Aqueous humor formation is driven by active ion transport across the ciliary epithelium, followed by osmotic water movement (30). Cl\textsuperscript{−} is the principal anion of the aqueous humor, and transepithelial Cl\textsuperscript{−} secretion likely plays a central role in aqueous humor formation (12, 14, 18, 51). Figure 1 illustrates a consensus model of aqueous humor formation. Cl\textsuperscript{−} can enter from the ciliary stroma into the PE cells through two main transcellular pathways: the Na\textsuperscript{+}–K\textsuperscript{+}–2Cl\textsuperscript{−} cotransporter and parallel Na\textsuperscript{+}/H\textsuperscript{+} and Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchangers (14, 18, 20, 35, 52). Cl\textsuperscript{−} can then diffuse through the intercellular gap junctions to the NPE cells and finally exit through Cl\textsuperscript{−} channels to the posterior chamber of the eye (11, 18, 54, 55). Many transport mechanisms underlying aqueous humor secretion have been identified, but the regulatory pathways remain elusive.

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saline (GIBCO-BRL, Grand Island, NY). The preparation was then incubated with 0.25% trypsin at 37°C for 30 min. Singly dissociated PE and NPE cells were obtained by trituration. Cells were washed twice with Dulbecco’s phosphate-buffered saline before they were seeded to the coverglass (Fisher Scientific, Pittsburgh, PA). Cells were incubated in medium 199 containing 10% fetal bovine serum and 0.1% gentamicin (GIBCO-BRL) at 37°C in 5% CO₂ before use. Rounded PE cells, as noted by the presence of abundant pigment granules, were chosen for patch clamp and cell volume measurements after being incubated for at least 3 h but not >36 h. Because of the absence of the tight junctions in PE cells (Fig. 1), channels patched either on the basolateral or apical surfaces were expected to have identical effects on transepithelial transport across the ciliary epithelium. In addition, pigment granules were generally localized along the apical border of the PE cells; the area less heavily pigmented was always chosen for patching.

**Patch clamp measurements.** Micropipettes for excised patches and whole cell configurations with resistances of 5–10 and 2–4 MΩ, respectively, were prepared from Corning glass (cat. no. 7052; World Precision Instruments, Sarasota, FL) using a Flaming/Brown micropipette puller (model P-97; Sutter Instruments). Micropipettes were re-polished with an IC-200 charge-coupled device camera (Photon Technology International, Foster City, CA). Analysis was conducted by using Axon Instruments Clampfit 8.2 software.

**Cell volume measurements.** Cell volume was monitored by measuring cell area using calcein fluorescence (37). Coverglasses were mounted in a chamber connected to a Nikon Diaphot microscope. Cells were washed with 4 μM calcein-AM and 0.02% Pluronic for 40 min and then perfused with isotonic Tyrode’s solution for 30 min before initiating the experiment. Calcein was excited every 20 s at 488 nm, and light emitted at 520 nm was detected with an IC-200 charge-coupled device camera (Photon Technology International, Princeton, NJ). Cell area was determined from the number of pixels detected above threshold within the cell region. Results were analyzed by using Imagemaster software (Photon Technology International). Experiments were conducted at room temperature.

**Solutions and pharmacological agents.** For single-channel recordings, the baseline solutions for the micropipette and for the bath were identical and contained (in mM): 130 NaCl, 20 sucrose, 10 HEPES, 0.674 CaCl₂, 1.1 EGTA, and 0.1 GTP (290 mosmol/kgH₂O, pH 7.4). Cl⁻ concentrations were reduced by replacing NaCl with isosmolal amounts of sucrose. For whole cell measurements, the standard bathing solution contained (in mM): 110 NaCl, 15 HEPES, 2.5 CaCl₂, 1.2 MgCl₂, 4.7 KCl, 1.2 KH₂PO₄, 30 NaHCO₃, and 10 glucose (295–305 mosmol/kgH₂O, pH 7.3). Where appropriate, the Cl⁻ concentration in the micropipette solution was reduced to 30 mM by equimolar substitution of aspartate.

For cell volume measurements, the isotonic Tyrode’s bathing solution contained (in mM): 110 NaCl, 15 HEPES, 2.5 CaCl₂, 1.2 MgCl₂, 4.7 KCl, 1.2 KH₂PO₄, 30 NaHCO₃, and 10 glucose (295–305 mosmol/kgH₂O, pH 7.3). Solutions were made hypotonic by reducing the concentration of NaCl from 110 to 55 mM (240–250 mosmol/kgH₂O).

All chemicals were reagent grade. cAMP, 8-bromo-cAMP (8-Br-cAMP), 5-nitro-2-(phenylpropylamino)benzoxate (NPPB), and SITS were purchased from Sigma (St. Louis, MO). All solutions were filtered through a 0.22-μm Millipore filter before use.

**Statistics.** Data are presented as means ± SE, where n is the number of experiments. The statistical significance of the differences was evaluated by using either one-way ANOVA followed by the Student-Newman-Keuls test or Student’s paired t-test. P < 0.05 was considered statistically significant.
RESULTS

Activation of maxi-Cl\textsuperscript{−} channels by cAMP in excised inside-out and outside-out patches. Under baseline conditions in which both bathing and micropipette solutions contained 130 mM Cl\textsuperscript{−}/H\textsubscript{11002}, maxi-Cl\textsuperscript{−} channel activity was rarely observed in cell-attached patches, consistent with previous observations (38). Its occurrence was also infrequently detected on excision, accounting for \textasciitilde 20% of the preparations that subsequently demonstrated maxi-Cl\textsuperscript{−} channel activity in the presence of cAMP. However, when Cl\textsuperscript{−} channels were seen under baseline conditions, the conductance was large (\textasciitilde 280 pS), corresponding to values reported for maxi-Cl\textsuperscript{−} or voltage-dependent anion channels (VDAC) (6, 27, 33, 44). Although the activity of maxi-Cl\textsuperscript{−} channels was not always detected under baseline conditions, the addition of cAMP to the cytoplasmic solution activated the maxi-Cl\textsuperscript{−} channels reversibly, displaying a linear conductance of 272 \pm 2 pS (\textit{n} = 80). Figure 2 shows the activation of maxi-Cl\textsuperscript{−} channel by cAMP in excised inside-out patches. No channel activity was observed either before adding or after removing cAMP. A: before adding cAMP. B: during cAMP exposure. C: after removing cAMP.

Fig. 2. Activation of maxi-Cl\textsuperscript{−} channels by cAMP (500 \textmu M) in an excised inside-out patch from native bovine PE cells. The holding potential (\textit{V\textsubscript{h}}) was 0 mV, and patches were clamped at membrane potentials (\textit{V\textsubscript{m}}) of \textasciitilde 80 to +80 mV in 20-mV steps. The channel was usually open when \textit{V\textsubscript{m}} was within \pm 40 mV; voltage-dependent channel inactivation was observed when \textit{V\textsubscript{m}} was beyond this range. The dotted and solid lines indicate closed (c) and open (o) states of the channel, respectively. Upward current deflections indicated inward currents, and vice versa. No channel activity was observed either before adding or after removing cAMP.

Fig. 3. \textit{V\textsubscript{m}}-dependence of open probability (\textit{P\textsubscript{o}}) for maxi-Cl\textsuperscript{−} channels in the presence of 500 \textmu M cAMP. Averages have been obtained from all patches that displayed open events at all applied voltages. The channel displayed \textit{V\textsubscript{m}}-dependent inactivation, especially when \textit{V\textsubscript{m}} was either greater than +40 mV or smaller than \textasciitilde 40 mV. The uppermost curve represented the baseline conditions in which Cl\textsuperscript{−} concentrations in the micropipette and bath were 130 mM. Reducing the cytoplasmic Cl\textsuperscript{−} concentration from 130 mM to either 65 or 30 mM reduced the \textit{P\textsubscript{o}} at all potentials. In all cases, the extracellular NaCl concentration was maintained constant at 130 mM, whereas the cytoplasmic Cl\textsuperscript{−} concentration was varied. The curves were fitted by two Boltzmann equations.
studies (6, 28, 39). It was noted that the plot is not symmetrical; $P_o$ was always higher at positive than at negative $V_m$, especially at extreme $V_m$, as described for other cell types (4, 16).

The addition of cAMP had no effect on the channel unitary conductance, changing from 277 ± 4 to 275 ± 4 pS on the addition of cAMP in paired measurements ($n = 25$). Instead, cAMP activated the maxi-Cl$^-$ channels primarily by increasing $P_o$. In symmetrical 130 mM NaCl solutions, cAMP caused a concentration-dependent increase in $P_o$ over the concentration range from 30 to 500 μM (Fig. 4), leading to a corresponding increase in mean patch current (Fig. 5). The cAMP-activated maxi-Cl$^-$ channel of the excised patches was significantly inhibited by Cl$^-$ channel blockers (Fig. 6). SITS (1 mM) produced a complete inhibition of maxi-Cl$^-$ channel in 4 of 5 experiments, and the remaining preparation showed a flickery inhibition at all potentials. Like SITS, NPPB (100 and 500 μM) caused a flickery/complete inhibition of cAMP-activated maxi-Cl$^-$ channels. In most cases, the inhibition was totally reversible.

The presence of maxi-Cl$^-$ channels was also detected in outside-out patches although its occurrence was considerably lower than that of inside-out patches. Like excised inside-out patches, the addition of the plasma-membrane permeable cAMP-analog 8-Br-cAMP (500 μM) to the bath activated the maxi-Cl$^-$ channel in symmetrical 130 mM NaCl solutions. The channel displayed a conductance of 271 ± 4 pS ($n = 14$), which was not different from that of the inside-out patches. Similarly, the addition of 100 μM NPPB significantly inhibited the maxi-Cl$^-$ channels by decreasing $P_o$ (data not shown). Taken together, our results indicated the presence of cAMP-activated maxi-Cl$^-$ channels in PE cells.

**Effects of cytoplasmic Cl$^-$ concentrations on maxi-Cl$^-$ channel activity.** As noted above, PE cells are primarily involved in Cl$^-$ uptake, thereby increasing the intracellular Cl$^-$ concentration. The increased Cl$^-$ might either diffuse to the NPE cells for subsequent Cl$^-$ secretion into the posterior chamber or return to the ciliary stroma through Cl$^-$ efflux pathways. We tested whether the intracellular Cl$^-$ concentration itself might modify the behavior of the cAMP-activated maxi-Cl$^-$ channels. This was done by reducing cytoplasmic NaCl concentration of inside-out patches from 130 to either 65 or 30 mM while maintaining extracellular NaCl concentration constant at 130 mM. Decreasing cytoplasmic Cl$^-$ concentration from 130 to either 65 or 30 mM caused a stepwise inhibition of the maxi-Cl$^-$ currents in the presence of cytoplasmic 500 μM cAMP. The inhibition could be explained partly by a reduction of $P_o$ at all potentials. The inhibition was, however, more pronounced when $V_m$ was negative. The plots of $P_o$ as a function of $V_m$ under different cytoplasmic Cl$^-$ concentrations (130, 65, and 30 mM) are summarized in Fig. 3. In addition to the reduction of $P_o$, cytoplasmic Cl$^-$ concentration reduced channel conductance. The decrease in Cl$^-$ channel conductance was observed with both inward and outward currents, the reduction being greater for inward currents (Fig. 7). Reducing cytoplasmic Cl$^-$ concentration from 130 to 30 mM lowered Cl$^-$ conductance by ~30% when $V_m$ was positive, whereas the reduction was ~70% when $V_m$ was negative. Taken together, decreasing the cytoplasmic Cl$^-$ concentration caused a significant inhibition of maxi-Cl$^-$ channels in excited
inside-out patches, which could be ascribed to reductions in both $P_o$ and channel conductance.

Reducing cytoplasmic NaCl concentration shifted the reversal potential, in addition to inhibiting maxi-Cl$^-$-channel activity. As shown in Fig. 7, the reversal potential was close to zero ($-0.7$ mV) in excised inside-out patches under symmetrical 130 mM NaCl but shifted to $-9.1$ and $-15.0$ mV when the cytoplasmic NaCl concentration was reduced to 65 and 30 mM, respectively. The negative shift of the reversal potential toward the Cl$^-$ equilibrium potential indicated that the channel was $\sim 3.0$ times more permeable to Cl$^-$ than Na$^+$. Taking into consideration the changes in junction potential, the selectivity was estimated to be $\sim 6$. The present estimate is comparable to the values of 3.7 to 10 reported by many investigators (5, 28, 29, 39, 45), but higher selectivities have also been reported (3, 22, 44). The basis for this broad range of apparent selectivities is unclear.

Stimulation of whole cell Cl$^-$ currents by 8-Br-cAMP. To learn whether cAMP-activated maxi-Cl$^-$ channels contributed significantly to PE-cell macroscopic currents, we studied the effects of 8-Br-cAMP and of the Cl$^-$ channel blocker NPPB on whole cell current measurements. In symmetrical 140 mM NMDGCl, the addition of 500 μM 8-Br-cAMP reversibly stimulated the whole-cell currents at all potentials (Fig. 8A). It was noted that the cAMP-activated currents displayed less outward rectification than did the baseline currents (Fig. 8B), suggesting that the cAMP-activated conductances were different from the baseline Cl$^-$ conductances. These measurements were obtained with our standard whole cell protocol involving step changes in voltage of relatively brief duration (300 ms). For comparison with the single-channel records, we also measured whole cell currents with voltage steps of longer duration. With the prolonged voltage pulses of 4 s, the cAMP-activated whole cell currents showed significant current inactivation at both $+80$ (P < 0.01) and $-80$ mV (P < 0.05) but not at $-40$ and $+40$ mV (P > 0.05, n = 12). A typical experiment showing the voltage-dependent whole cell current inactivations is shown in Fig. 9. This is consistent with the channel inactivation due to $V_m$-dependent closing of maxi-Cl$^-$ channels as displayed in excised inside-out patches (Fig. 3). These results strongly suggest that the cAMP-activated Cl$^-$ channels observed with excised patches contribute currents that can be detected by macroscopic measurements, as well.

Regulation of whole cell cAMP-activated Cl$^-$ currents by cytoplasmic Cl$^-$ concentration. As demonstrated earlier, the activities of cAMP-activated maxi-Cl$^-$ channels were modified by the Cl$^-$ concentration at the cytoplasmic surface of excised patches (Figs. 3 and 7). We tested whether the stimulation of cAMP-activated whole cell currents is also sensitive to changes of cytoplasmic Cl$^-$ concentration. At constant bath Cl$^-$ concentration of 140 mM, reducing micropipette (cytoplasmic) Cl$^-$ concentration from 140 to 30 mM decreased the magnitude of cAMP-activated currents (Fig. 10). When the cytoplasmic solution contained 140 mM Cl$^-$, the inward current was increased by $48 \pm 6\%$ over the baseline values at $V_m = -80$ mV (n = 14). The stimulation was approximately twofold larger than a separate set of experiments with 30 mM Cl$^-$ in the micropipette (25 ± 4%, n = 6). In both cases, the addition of NPPB to the bath completely blocked the cAMP-activated current stimulation. Similarly, the stimulation of outward current was approximately twofold larger when higher cytoplasmic Cl$^-$ concentration was used, increasing from 14 ± 2% (30 mM Cl$^-$, n = 6) to 34 ± 5% (140 mM Cl$^-$, n = 14) at $+80$ mV. These results indicated that the cAMP-activated whole cell Cl$^-$ conductance could be modulated by cytoplasmic Cl$^-$ concentration, consistent with the results from excised inside-out patches.

cAMP-triggered shrinkage of PE cells. The electrophysiological data indicated that cAMP activated Cl$^-$ channels so that we wondered whether cAMP-activated Cl$^-$ efflux was sufficiently great to produce volume changes in the PE cells. Under isotonic conditions, the addition of 8-Br-cAMP (500 μM) to the extracellular bath produced a gradual, time-dependent shrinkage of PE cells (Fig. 11). 8-Br-cAMP shrank cells by 6.3 ± 1.3% over a period of 25 min (n = 18). We tested whether the shrinkage was mediated by Cl$^-$ release through cAMP-activated Cl$^-$ channels. In a separate set of experiments, 100 μM NPPB significantly blocked cAMP-triggered PE cell shrinkage; in the presence of the Cl$^-$-channel blocker, cells shrank by 0.9 ± 1.5% by 25 min (n = 13). Subsequent removal of NPPB from these preparations restored cAMP-triggered cell shrinkage similar to that produced in cells without preexposure to NPPB (data not shown).

DISCUSSION

The major new findings of the present study are as follows: 1) cAMP reversibly activates maxi-Cl$^-$ channels of excised patches from native bovine PE cells, 2) the Cl$^-$ concentration on the cytoplasmic surface triggers modification in these channels, and 3) the cAMP-activated maxi-Cl$^-$ channels transfer sufficient Cl$^-$ to contribute significantly to whole cell currents and participate in cell-volume regulation.

Maxi-Cl$^-$ channels share many similarities with VDAC including unitary channel conductance, multiple subconductance states, linear current-voltage (I-V) relationship, voltage dependence, anion selectivity, and sensitivity to Cl$^-$ channel blockers (6, 33, 42, 44). The molecular identity of maxi-Cl$^-$

Fig. 7. Effects of reduced cytoplasmic Cl$^-$ concentration on cAMP-stimulated unitary single-channel currents. In the presence of cytoplasmic cAMP (500 μM), the concentration of NaCl at the cytoplasmic side of inside-out patches was reduced from 130 mM to either 65 or 30 mM, whereas the extracellular NaCl concentration was kept constant at 130 mM. Data points are usually means of 6–12 patches, because channels were not always open long enough for precise unitary-current measurement at all potentials.
channels is likely porin-1 (VDAC-1, porin-31HL), which has been localized in the plasma membrane of cultured T-lymphoblastic leukemia CEM cells (2) and astrocytes (15). Although plasma-membrane maxi-Cl\(^{-}\)/H\(^{+}\) channels have been recognized in many cells (6), including PE cells (38) for two decades, their physiological roles have been uncertain (1, 44, 47, 50), primarily because of the voltage-dependence of their activity. Their maximum \(P_{o}\) is displayed within a narrow voltage range (−20 to +20 mV), far from the \(V_{m}\) of many cells.

It has been shown that \(V_{m}\) of both PE and NPE cells is approximately −60 mV in rabbit and shark ciliary epithelium (9, 26, 53). A membrane-permeant form of cAMP, 8-Br-cAMP, was reported to depolarize porcine ciliary epithelium by ∼10 mV (23). That action could reflect activation of Cl\(^{-}\) channels of either the NPE (10, 11, 20) or of PE cells (24).

Consistent with the observation in transformed cultured cells (24), we have now documented that 8-Br-cAMP activates Cl\(^{-}\) channels of native bovine PE cells, facilitating Cl\(^{-}\) release and thereby reducing the cell volume. At least one of the channel targets is the maxi-Cl\(^{-}\) channel and the activation is likely to be direct, because perfusion with cAMP activates the channels in excised patches. Interestingly, cAMP has been recently reported (16) to have no effect on baseline whole cell currents of C1300 mouse neuroblastoma cells but to block the antiestrogen-induced activation of maxi-Cl\(^{-}\) channels; the inhibitory effect of cAMP was prevented by staurosporine. The basis for the diverse effects of cAMP on the two cell types is not apparent but might reflect differential expression of two distinct pathways for cAMP to modulate maxi-Cl\(^{-}\) channels, either by direct activation or by indirect inhibition through protein kinases (49).

Several lines of evidence suggest that the cAMP-activated maxi-Cl\(^{-}\) channels observed with excised patches contribute significantly to PE whole cell currents. First, both unitary maxi-Cl\(^{-}\} and whole cell currents are stimulated by the addition of cAMP, and the effects are prevented by NPPB. Second, measured as the mean value of initial 300 ms, cAMP-stimulated whole cell currents displayed a more linear \(I-V\) relationship than did baseline currents, consistent with the linear relationship displayed by cAMP-activated maxi-Cl\(^{-}\) channels. Third, the cAMP-activated unitary maxi-Cl\(^{-}\} and whole cell currents are both dependent on cytoplasmic Cl\(^{-}\) concentration,

![Fig. 8. Effects of the cell membrane-permeable cAMP analog 8-bromo-cAMP (8-Br-cAMP; 500 μM) and of the Cl\(^{-}\)-channel blocker NPPB (100 μM) on whole cell currents. A: representative experiment showing the effects of 500 μM 8-Br-cAMP on whole cell currents. Voltage pulses were from −100 to +80 mV in 20-mV steps, and each voltage pulse lasted 300 ms. Upward current deflections indicated outward currents and vice versa. The Cl\(^{-}\) concentrations in the micropipette and in the bath were 140 mM. B: Current-voltage (I-V) relationship generated from the experiment in (A).](http://ajpcell.physiology.org/doi/fig/10.1152/ajpcel.00008.2004)
Fig. 9. Time courses of whole cell cAMP-stimulated currents following step changes in $V_m$. Voltage pulses were from −80 to +80 mV in 40-mV steps and each voltage step was 4 s, as in excised patches ($V_h = 0$ mV). The Cl$^-$ concentrations in the micropipette and in the bath were identical (140 mM). A: representative experiment showing the time-dependent whole cell cAMP-stimulated currents inactivating at −80 and +80 mV but not at −40 and +40 mV. B: $I$-$V$ relationship from the experiment in (A). Currents were calculated from the mean values of the initial 500 ms (early endpoint) and also from the last 500 ms (late endpoint) in each 4-s voltage pulse at different $V_m$.

Fig. 10. Effect of cytoplasmic Cl$^-$ concentration on cAMP-activated whole cell currents. Pulses were of brief duration (300 ms) at $V_m = −80$ mV. The Cl$^-$ concentration in the external bath was always 140 mM. Currents have been normalized and averaged. Mean ± SE values are shown by vertical bars. The absolute baseline currents with 30 and 140 mM NaCl were $72 ± 10$ and $−70 ± 15$ pA·pF$^{-1}$ ($P = 0.96$), respectively. **$P < 0.01$.

Fig. 11. Effects of 8-Br-cAMP on PE-cell volume. 8-Br-cAMP (500 μM) triggered a gradual, time-dependent shrinkage of native bovine PE cells under isosmotic conditions ($n = 18$). The SE values are shown by vertical bars. *$P < 0.05$; **$P < 0.01$. 

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displaying an increase of currents at higher intracellular Cl− concentration. Fourth, time- and voltage-dependent current inactivations are observed at Vm of ±80 mV and not at ±40 mV in both excised single-channel and whole cell measurements, consistent with the typical characteristics of maxi-Cl − channels. In addition to maxi-Cl − channels, it is possible that cAMP enhances whole cell currents by activating other Cl− channels in PE cells. However, if an additional channel is targeted, it is unlikely to be CFTR, because Northern blot analysis has not detected mRNA for CFTR in human ciliary body (13). Moreover, patients with cystic fibrosis demonstrate normal aqueous flow rate compared with healthy subjects (34).

The cAMP-activated maxi-Cl − channels observed in the present study were strongly influenced by cytoplasmic Cl − concentration. Raising intracellular Cl − concentration enhanced both inward and outward currents by a dual effect on P0 and channel conductance. Under physiological conditions, the PE-cell cytoplasmic Cl − concentration is determined by the rate of uptake from the stroma by electroneutral transporters and the rate of Cl − diffusion through gap junctions to the NPE cells and final release into the aqueous humor. Thus the Cl − concentration is expected to rise when the rate of PE-cell Cl − uptake from the stroma exceeds the NPE-cell transport capacity to secrete Cl − into the aqueous humor. The rise in PE-cell Cl − concentration, accompanied by a rise in the counter-ion Na+, is predicted to present an osmotic gradient for water and cell swelling. Hypotonic swelling has been found to trigger NPPB-sensitive ATP release from cultured bovine PE cells (36). The ATP can occupy P2Y2 receptors, initiating sequential increases in free Ca2+ concentration, phospholipase A2 activity, PGE2 formation and release, occupancy of PGE2 receptors, formation of cAMP in Madin-Darby canine kidney epithelial cells (40), and transformed bovine PE cells (24). In other words, Cl −-overloaded PE cells would be expected to swell, triggering a cascade of events leading to cAMP formation, and thereby activating the maxi-Cl − channels directly. In agreement with the electrophysiological measurements, cAMP triggers a reduction of cell volume, which can be prevented by pretreatment with NPPB. Increased fluid transfer from the PE cells to the ciliary stroma is expected to reduce net aqueous humor secretion.

Although Cl − overload could provide one source of delivery of cAMP to the PE cells through an ATP-release-triggered cascade, other mechanisms may prove of even greater physiological importance. For example, PE cells express β2-adrenergic receptors (21) so that sympathetic nerve endings located close to the ciliary epithelium in the ciliary processes could stimulate cAMP production in these cells. Multiple biologically active peptides are also thought to be released from those nerve endings (48). Included among the neupeptides are VIP and substance P, both of which trigger increased production of cAMP (e.g., see Refs. 31 and 43).

The focus of the current work has been on the cAMP activation of the PE cell maxi-Cl − channels and their potential physiological role in modifying net Cl − secretion by the ciliary epithelium. However, strong published evidence indicates that maxi-Cl − channels also mediate swelling-triggered ATP release by mouse mammary C127 cells (19, 44). Consistent with their findings, and in contrast to the cAMP insensitivity of release by transformed bovine PE cells (36), we have recently found that 8-Br-cAMP (500 μM) enhances isotonic ATP release by native bovine PE cells by ~50% (Do CW, Reigada D, Mitchell CH, and Civan MM, unpublished data). The potential role of maxi-Cl −-mediated ATP release in regulating aqueous humor formation is currently under study.

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