PGE<sub>2</sub>, Ca<sup>2+</sup>, and cAMP mediate ATP activation of Cl<sup>-</sup> channels in pigmented ciliary epithelial cells

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AQUEOUS HUMOR SECRETION is a determinant of intraocular pressure, so that reducing the secretory rate is a major strategy in treating glaucomatous patients. The aqueous humor also delivers substrates, oxygen, and the antioxidant ascorbate to the avascular cornea, lens, and trabecular meshwork, removes metabolic waste products, and facilitates immune responses (20). The bilayered ciliary epithelium forms aqueous humor by transferring solute (and, secondarily, water) from the stroma of the ciliary processes to the contralateral posterior chamber of the eye. Solute is taken up from the stroma by the pigmented ciliary epithelial (PE) layer, passes through gap junctions to the nonpigmented ciliary epithelial (NPE) layer, and is then released into the aqueous humor.

Several lines of evidence suggest that Cl<sup>-</sup> channel activity limits the rate of secretion (7). Activating Cl<sup>-</sup> channels of the NPE cells is expected to increase secretion, whereas activating Cl<sup>-</sup> channels of the PE cells should favor reabsorption, thereby reducing net secretion. Purines may regulate activity of Cl<sup>-</sup> channels on both sides of the tissue. At the aqueous surface of the epithelium, A<sub>2a</sub>-subtype adenosine agonists activate Cl<sup>-</sup> channels (5, 25). At the stromal surface, ATP and the estrogen receptor antagonist tamoxifen synergistically activate Cl<sup>-</sup> channels of bovine PE cells (26). The aim of the present study was to examine the effect of ATP itself on these cells.

The strategy of the study was to focus on ATP-triggered transfer of fluid out of PE cells with volumetric measurements and to verify the role of Cl<sup>-</sup> channels by patch clamping. These approaches were supplemented by luciferin/luciferase assay of ATP release in addressing the identity of the nucleotide receptor(s) involved.

METHODS

**Cellular model: Bovine PE cells.** We have extended our studies of an immortalized PE cell line developed by M. Coca-Prados from a primary culture of bovine PE and characterized by several investigators (10, 26, 34). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; no. 11965–084, Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (SH30071.03, HyClone Laboratories, Logan, UT) and 50 μg/ml gentamicin (no. 15750–060, Gibco-BRL), at 37°C in 5% CO<sub>2</sub> (36). The medium had an osmolality of 328 mosmol/kgH<sub>2</sub>O. Cells were passaged every 6–7 days.

**Volumetric measurements and analysis.** After reaching confluence, cells from a T-75 flask were harvested by trypsinization within 3–10 days after passage (8). 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

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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) with a 100-µm aperture. As previously described, the cell volume (Vc) of the suspension was taken as the peak of the distribution function. The time course of cell shrinkage was fit to a monoexponential by nonlinear least-squares analysis, and the probability of the null hypothesis (that any 2 sets of observations were derived from the same population) was obtained from the F distribution (10).

Whole cell patch-clamp recording. Micropipettes were pulled from Corning no. 7052 glass, coated with Sylgard, and fire polished. The resistances of the micropipettes in the bath usually ranged from ~1.0 to 2.6 MΩ; successful seals displayed gigaohm resistances. Unless otherwise stated, currents were recorded in the ruptured-patch mode. After rupture of the membrane patch, the series resistance was measured to be only 8.1 ± 0.7 MΩ and was therefore not usually compensated; whole cell capacitance was 10.4 ± 1.2 pF. The baseline whole cell currents were 83 ± 17 pA/pF. In a subset of experiments (n = 8), we measured whole cell currents in the perforated-patch mode. In those experiments, we back-filled the micropipettes with solution containing amphoterocerin (168 µg/ml) and filled the tips with amphoterocerin-free solution (1). The applied voltages were not corrected for the small junction potentials (approximately −2.8 mV; Ref. 6) arising from the present micropipette filling and external solutions.

Data were acquired at 2−5 kHz with either an Axopatch 1D (Axon Instruments, Foster City, CA) or a List L/M-EP-C7 (Darmstadt, Germany) patch-clamp amplifier and filtered at 500 Hz. The membrane potential was held at −40 mV and stepped to test voltages from −100 to +80 mV in 20-mV increments at 1-s intervals. Each step lasted 300 ms with intervening periods of 1.7 s at the holding potential. Stimulatory responses were measured at peak levels and inhibitory responses at the nadirs.

ATP measurements. Bovine PE cells were grown for 4–48 h to confluence on glass coverslips. Cells were washed in control solution and mounted on an inverted microscope, and bath ATP levels were measured continuously by including 2 mg/ml of luciferin/luciferase assay mixture (33). After background levels were recorded, a solution containing luciferin/luciferase assay mixture and either control solution or UTP was carefully added to the cells. ATP released from cells into the extracellular bath reacted with the luciferase and led to luminescence in the presence of the luciferin/luciferase assay mixture. The control solution contained (in mM) 105 NaCl, 4.5 KCl, 2.8 Na-HEPES, 7.2 HEPES acid, 1.3 CaCl2, 0.5 MgCl2, 5 glucose, and 75 mannitol. The pH was adjusted to 7.4 with NaOH, and the solution had an osmolality of 304–312 mosmol/kgH2O.

Chemicals. All chemicals were reagent grade. Gramicidin D, ionomycin, tamoxifen, ATP, UTP, ADP, UDP, GTP, 2-methylthio-ATP, 8-bromoadenosine 3’,5’-cyclic monophosphate (8-BrcAMP), dibutyryl cAMP (DBcAMP), and indomethacin were purchased from Sigma (St. Louis, MO); 4,4’-Dispirothiocyanato-

Fig. 1. Effects of ATP on cell volume. ATP triggered concentration-dependent shrinkage of PE cells at 10 and 100 µM but not at 3 µM (Fig. 1A). Test solutions in Fig. 1A contained the cation ionophore gramicidin (5 µM) to incorporate an exit port for K+ in the plasma membrane. Under these conditions, the observed shrinkage reflected activation of a Cl− release pathway (8). ATP also produced comparable shrinkage without gramicidin (Fig. 1B). As previously reported (26), ATP did not uniformly trigger shrinkage; no response was noted in ~20% of the present series of volumetric

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measurements. Averaging the results of 11 series of experiments (reflecting 43 experiments), ATP produced a magnitude of shrinkage ($\Delta V_{\text{m}}$) of 4.2 $\pm$ 0.3% with a time constant ($\tau$) of 5.2 $\pm$ 0.6 min. In controls (14 series, 50 experiments), $\Delta V_{\text{m}} = 1.3$ $\pm$ 0.3% and $\tau = 9.5$ $\pm$ 2.5 min (in 10 series of controls with significant shrinkage). In agreement with our previous study (26), tamoxifen uniformly enhanced the response to ATP ($n = 10$, Fig. 2B). In contrast, adenosine has no significant effect on the volume of these PE cells with or without tamoxifen (26).

The ATP-triggered shrinkage was inhibited by Cl$^-$ channel blockers. When tested in parallel aliquots of cell suspensions, NPPB (100 $\mu$M) and DPC (1 mM) were similarly effective in blocking shrinkage (Fig. 1B).

UTP and UDP (Fig. 2A) and ADP (Fig. 2B) also triggered shrinkages. These effects were smaller than the ATP-triggered shrinkage, precluding a definitive ranking of the effects of ADP, UDP, and UTP.

Effect of nucleotides on PE whole cell currents. ATP altered whole cell currents in approximately one-third of the bovine PE cells (see Table 2). Two different electrophysiological effects were noted, sometimes in the same cells (Fig. 3). The stimulatory effect is exemplified by the increase in whole cell currents beginning 1 min after initiation of perfusion with 10 $\mu$M ATP (Fig. 3A). Raising the external ATP concentration to 100 $\mu$M then triggered the second characteristic effect, a small inhibition of outward current at $+80$ and $+60$ mV within $\sim$40s. The later rate of increase in whole cell currents was not detectably altered by this increase in ATP concentration. Application of the Cl$^-$ channel blocker NPPB (100 $\mu$M) subsequently inhibited the currents by $\sim$70%. The time courses of ATP-difference currents after step changes in voltage are presented in Fig. 3B, and the current-voltage relationships of the ATP- and NPPB-difference currents are presented in Fig. 3C. Slight inactivation was noted at highly depolarizing potentials (Fig. 3B); the magnitude of depolarization-induced inactivation displayed by Cl$^-$ channels of many cells depends on the free intracellular $\text{Mg}^{2+}$ concentration and other unidentified factors (29). The current-voltage relationship was outwardly rectifying (Fig. 3C).

The reversal potential for the ATP-activated currents (Fig. 3C) was $-26.8$ $\pm$ 1.8 mV. Taking into account a junction potential of approximately $-2.8$ mV estimated for similar filling and bath solutions (5), the corrected reversal potential was $-29.6$ mV. When this value and the known anionic concentrations inside and outside the cell (Table 1) are inserted in the Goldman

![Fig. 2. Relative effects of nucleotides on cell volume. A: fit values for $\Delta V_{\text{m}}$ and $\tau$ for UTP, UDP, and ATP ($n = 4$): control (1.9 $\pm$ 0.6%, 23.6 $\pm$ 15.5 min), 100 $\mu$M ATP (4.1 $\pm$ 0.2%, 4.8 $\pm$ 1.1 min; $P < 0.01$), 100 $\mu$M UTP (3.4 $\pm$ 1.2%, 31.6 $\pm$ 20.2 min; $P < 0.01$ vs. ATP, $P > 0.05$ vs. control), 100 $\mu$M UDP (2.7 $\pm$ 0.3%, 11.0 $\pm$ 2.8 min; $P < 0.01$ vs. both ATP and control). B: fit values for $\Delta V_{\text{m}}$ and $\tau$ for ADP, tamoxifen, and ATP ($n = 4$): control (1.5 $\pm$ 0.2%, 1.0 $\pm$ 1.3 min), 100 $\mu$M ADP (2.4 $\pm$ 0.5%, 7.3 $\pm$ 1.9 min; $P < 0.05$ vs. control, $P < 0.01$ vs. ATP), 100 $\mu$M ATP (5.6 $\pm$ 0.5%, 7.3 $\pm$ 1.9 min; $P < 0.01$), 10 $\mu$M tamoxifen + 100 $\mu$M ATP (not fit by single exponential, but data points significantly different from control, ATP, and ADP at $P = 0.01$).](http://ajpcell.physiology.org/doi/10.1152/ajpcell.00334.2000)
equation, the relative permeability of aspartate/Cl\textsuperscript{−} of the ATP-activated anion channels can be estimated (5) to be ~0.14.

Whole cell responses to UTP are illustrated in Fig. 4. As shown in Fig. 4A, 10 μM UTP produced small initial inhibitions of outward currents followed by stimulation of both outward and inward currents. The UTP-difference currents displayed the same characteristics observed with ATP-difference currents (Fig. 3): slight inactivation at highly depolarizing potentials (Fig. 4B) and outward rectification with a comparable uncorrected reversal potential (~33.4 ± 1.2 mV; Fig. 4C). As for the ATP experiments, the magnitudes of the responses to UTP are displayed in Fig. 5 and the frequency of the responses is given in Table 2. ADP and UDP triggered similar changes, but the magnitudes were smaller (Fig. 5, Table 2).

To summarize the electrophysiological results obtained with all nucleotides, the means ± SE of stimulatory and inhibitory responses to 10 and 100 μM concentrations are presented in Fig. 5 and the frequency with which these effects were observed is presented in Table 2. No response to ATP was seen after 1 or 3 μM ATP. At 10 and 100 μM concentrations, stimulations were seen in 25–45% of the cells studied. There was considerable variance in response, but the changes appeared generally larger after ATP than after UTP, ADP, and UDP (Fig. 5). Block was observed in a similar fraction of the cells studied, with UTP producing a larger effect than did the other three nucleotides (Fig. 5). For all nucleotides studied, the nonzero reversal potential, outward rectification, inactivation at highly depolarizing potentials, and sensitivity to NPPB established that the stimulated currents reflected activation of Cl\textsuperscript{−} channels.

Because <50% of the cells responded to nucleotides, we wondered whether dialysis of important components out of the cell could have limited the frequency of response. However, the response rate to ATP and UTP was not significantly enhanced by using the perforated-patch mode of whole cell recording. Stimulatory

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Osmolality, mosmol/kgH\textsubscript{2}O 290–300 275–280 295–305/250–260*
pH 7.4 7.2 7.4

*Depending on inclusion/omission of mannitol.
and inhibitory responses were observed in 5 and 3 of 11 cells, respectively.

Second messenger cascade assayed by shrinkage. ATP increases intracellular Ca\textsuperscript{2+} activity of bovine PE cells (26). Raising intracellular Ca\textsuperscript{2+} levels by adding ionomycin triggered PE cell shrinkage similar to that produced by ATP, and the two effects were not additive (Fig. 6A).

An increase in intracellular Ca\textsuperscript{2+} activity can in turn activate phospholipase A\textsubscript{2} (4), enhancing formation of prostaglandin (PG)E\textsubscript{2}. Indeed, 10 μM PGE\textsubscript{2} produced effects similar to those of Ca\textsuperscript{2+}, replicating the actions of 100 μM ATP. Application of ATP together with PGE\textsubscript{2} did not significantly enhance the action of PGE\textsubscript{2} alone (Fig. 6B). The effects of ionomycin and PGE\textsubscript{2} were also not additive (Fig. 7C).

Production and release of PGE\textsubscript{2} would in turn be expected to trigger cAMP formation by occupancy of EP\textsubscript{2} receptors known to be functionally expressed in bovine PE cells (2), so we tested whether cAMP could replicate the responses to ATP. 8-BrcAMP (500 μM) and 100 μM ATP produced similar degrees of shrinkage (Fig. 6C).

The foregoing data suggested that ATP might activate Cl\textsuperscript{−} channels, and thereby shrinkage, by stimulating increases in Ca\textsuperscript{2+}, PGE\textsubscript{2}, and cAMP, but it was unclear whether these second messengers were acting in parallel or in tandem, as recently found in Madin-Darby canine kidney (MDCK) cells (30). We addressed this issue by attempting to block increases in Ca\textsuperscript{2+} (with the Ca\textsuperscript{2+} buffer BAPTA), 2) in PGE\textsubscript{2} (with the cyclooxygenase inhibitor indomethacin), and 3) in cAMP-activated protein kinase activity (with the inhibitory Rp stereoisomer of 8-BrcAMPS).

Preincubation with BAPTA-AM for 1 h to buffer intracellular Ca\textsuperscript{2+} (18) exerted no direct effect on cell volume but completely abolished ATP-triggered shrinkage (Fig. 7A). In contrast, similar treatment with TPEN, a chelator of heavy metals other than Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, did not alter the subsequent response to ATP (Fig. 7A).

Even in the presence of BAPTA-AM, both 10 μM PGE\textsubscript{2} and 500 μM 8-BrcAMP triggered shrinkage (data not shown; n = 4; P < 0.01). We then examined whether blocking the cyclooxygenase pathway of arachidonic acid metabolism would also affect the response to ATP. As illustrated by Fig. 7B, both 1 and 100 μM indomethacin inhibited the ATP-triggered shrinkage. The indomethacin was not acting simply as a channel blocker. Suspending cells in solution containing 10 μM PGE\textsubscript{2} together with 100 μM indomethacin and 100 μM ATP overcame the inhibitory effects of each of these agents acting simply as a channel blocker. Suspending cells in solution containing 10 μM PGE\textsubscript{2} together with 100 μM ATP (Fig. 7B) and indomethacin (Fig. 7C) did not alter the subsequent response to ATP (Fig. 7A). The indomethacin was not acting simply as a channel blocker. Suspending cells in solution containing 10 μM PGE\textsubscript{2} together with 100 μM ATP (Fig. 7B) and indomethacin (Fig. 7C) did not alter the subsequent response to ATP (Fig. 7A).

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methacin inhibition (data not shown; \( n = 5; P < 0.01 \) compared with parallel aliquots suspended in indomethacin and ATP alone).

In our third approach, we focused on cAMP in trying to interrupt the signaling pathways initiated by ATP. In a preliminary test, we found that the specific form of cAMP used to activate shrinkage was not critical. Effects similar to those elicited by 8-BrcAMP, albeit faster, were triggered by the more permeable analog DBcAMP (500 \( \mu M \)) (Fig. 8A). Also, simultaneous addition of the two analogs produced the same response as did DBcAMP alone (Fig. 8A), so that 500 \( \mu M \) is maximally effective. Because cAMP commonly modifies channel activity through protein kinase A (PKA), we applied the inhibitory diastereoisomer (Rp) of 8-BrcAMP. Unexpectedly, Rp-8-BrcAMPs produced similar degrees of cell shrinkage (\( n = 6 \)). Fit values for \( \Delta v_c \) and \( t \): control (NS), 100 \( \mu M \) 8-BrcAMP (4.0 \( \pm 0.4 \%), 7.1 \pm 2.0 \min; P < 0.01), 100 \( \mu M \) Rp (3.2 \( \pm 0.2 \%), 3.2 \pm 0.7 \min; P < 0.01), 100 \( \mu M \) Sp (4.4 \( \pm 0.4 \%), 6.0 \pm 1.8 \min; P < 0.01).

DBcAMP is not the same as ATP, and adding Rp-8-BrcAMPs and ATP together had the same effect as adding ATP alone (Fig. 8B). Parallel
additions of the inhibitory diastereoisomer, the stimulatory isomer Sp-8-BrcAMPS, and 8-BrcAMPS in the same series of experiments elicited similar shrinkages (Fig. 8C).

**Second messenger cascade assayed by whole cell currents.** Perfusion with 10 μM PGE2 activated Cl− currents in five of five cells by 88 ± 27% (+80 mV; P < 0.01). In the experiment shown in Fig. 9A, perfusion with either 1 mM DPC or 100 μM NPPB reversibly inhibited the activated currents and isotonic washout was associated with decay of the currents. The time courses of the PGE2-difference currents after step changes in voltage are displayed in Fig. 9B, and the current-voltage relationships are presented in Fig. 9C. With 100 μM indomethacin present to block PGE2 production, 100 μM ATP produced no stimulatory response (n = 3), and, conversely in the presence of 100 μM ATP, indomethacin reduced current by 31 ± 8% (+80 mV; n = 4; P < 0.05); one-half of these cells displayed a stimulatory response to the ATP pretreatment.

The effects of 8-BrcAMP on Cl− channels were also examined with whole cell patch clamping. In the experiment shown in Fig. 10A, 100 μM 8-BrcAMP increased outward and inward currents. Raising the concentration to 500 μM further stimulated the currents, producing a cumulative stimulation of ~80% at +80 mV. NPPB subsequently inhibited the activated currents reversibly by >90%. The time courses of the difference currents after step changes in voltage and the current-voltage relationships are displayed in Fig. 10B and C, respectively. At concentrations of 100 and 500 μM, 8-BrcAMP stimulated currents in four of nine experiments without exerting any blocking effect (Fig. 5).

Similar to the ATP- and UTP-triggered responses, the PGE2 (Fig. 9B) and 8-BrcAMP (Fig. 10B)-difference currents displayed slight inactivation at highly depolarizing potentials (Fig. 8B) and outward rectification (Figs. 9C, 10C) with similar uncorrected reversal potentials (~31.2 ± 3.3 mV in Fig. 9C and ~32.7 ± 2.0 mV in Fig. 10C).

**P2 receptors.** If a single P2 receptor modulated Cl− channel activity in PE cells, the data of Figs. 2 and 5 might point to P2Y11 (see DISCUSSION). Because the bovine sequence for this receptor is unknown, we conducted a functional test for its presence. The EC50 values of 2-methylthio-ATP at turkey P2Y1 and human P2Y11 receptors are 6 nM and 50 μM, respectively (17). However, neither 100 nM nor 100 μM concentrations of 2-methylthio-ATP triggered the shrinkage produced by 100 μM ATP (n = 4; data not shown).

**ATP release by PE cells.** Multiple complexities limit the identification of P2 receptors affecting cell func-
sampling 50,000 cells or more for each data point. By this measure and at a concentration of 100 μM, the ranking is ATP > UTP, ADP, UDP (Fig. 2).

To place the nonuniformity of response to ATP in perspective, we note that previous investigators reported a nonuniform expression for other channels in studies of PE cells. For example, only ~15% of fresh and cultured bovine PE cells exhibit T-type Ca^{2+} channels (16) and 22% of rabbit PE cells display L-type Ca^{2+} channels (13). Fain and Farahbakhsh (12) found voltage-gated Na⁺ channels in ~25% of the primary cultures of rabbit PE cells they studied. Stelling and Jacob (32) reported that carbachol increased K⁺ conductances in only ~30% and Cl⁻ conductance in 49% of the freshly dissected bovine PE cells they studied. Mitchell et al. (27) also found that GTPγS activated two-thirds of large-conductance Cl⁻ channels in patches taken from freshly dissected bovine PE cells ultimately shown to possess such a channel. Given the syncytial nature of the bilayered ciliary epithelium (7), activation of the Cl⁻ channels in 25–45% of the PE cells should provide a physiologically significant pathway for release of solute to the stroma.

Nucleotide receptors. Identification of functionally important receptor(s) is generally even more complex for P2 than for P1 (adenosine) receptors because (11, 21) 1) specific agonists and antagonists are not available; 2) the ectoenzymes apyrase, ecto-ATPase, ecto-ADPase, 5'-nucleotidase, and ectonucleoside diphosphokinase not only metabolize adenine nucleotides to adenosine but also interconvert purine and pyrimidine nucleotides; 3) cells frequently possess multiple P2 and P1 receptors, which can exert opposing effects; and 4) the final functional effects can be highly dependent on the specific cell studied because of synergistic interactions of the second messenger cascades. An additional caveat is illustrated by Fig. 11. Consistent with obser-

**Fig. 11.** UTP triggers release of ATP. After ~4 min in control solution, the bath was changed to a control solution containing luciferin/luciferase. After a further ~10 min, the solution was replaced with UTP containing luciferin/luciferase, which triggered a clear increase in extracellular ATP concentration. The vertical lines at ~5 and ~15 min represent room light reaching the chamber during solution changes. Solid data lines represent mean responses, and dotted lines show associated SE (n = 10).

**DISCUSSION**

**Initial observation.** External nucleotides activated Cl⁻ channels and triggered release of intracellular solute and water from bovine PE cells. On average, ATP produced larger stimulations of Cl⁻ currents than did UTP, ADP, and UDP at the same concentration (Fig. 5). However, the observation that more than half the cells did not respond and the ranges in magnitude of the responses made it difficult to rank the nucleotide responses from the patch-clamp data. In this respect, electronic cell sorting provided a more feasible approach, directly measuring the parameter of central interest (the transfer of fluid out of the cells) and
vations in other cells (9, 23, 30), application of one triphosphate nucleotide can trigger release of another nucleotide from intracellular stores. The concentrations of ATP measured directly in this study, although probably an underestimate, should be sufficient to stimulate the P2Y2 receptor with an EC50 of 200 nM (22).

Despite these caveats, two general conclusions can be drawn from the data. First, the dominant functional receptors must be P2Y metabotropic, rather than P2X ionotropic receptors. Otherwise, ATP activation of P2X cation-selective channels would have triggered influx of cation, leading to swelling of the PE cells, contrary to observation. Second, it is unlikely that the nucleotide-triggered shrinkage was mediated by occupancy of a single population of P2Y receptors. Of the six cloned functional P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, and P2Y12; Refs. 11 and 14), only P2Y11 might conform to the nucleotide ranking displayed by Fig. 2. Our inability to detect a response to the P2Y11 agonist 2-methylthio-ATP at 100 μM suggests that this receptor is not playing a dominant role in mediating the responses to ATP. We conclude that the transport effects of the nucleotides likely reflect occupancy of multiple P2 receptors of the PE cells. This conclusion is consistent with the reported detection of at least two different P2Y receptors in bovine PE cells (31, 34).

Second messengers. The present data suggest a plausible signaling cascade. We have found that elevation of intracellular Ca2+ activity and separate application of PGE2 and 8-BrcAMP all mimic ATP in reducing cell volume, and the effects of these agents are not additive with those of ATP. In principle, all three second messengers could act in parallel to mediate the ATP-triggered activation of Cl– channels (Fig. 12A). Alternatively, ATP could trigger a cascade involving the sequential activation of the three second messengers (Fig. 12B). More complex pathways involving both parallel and series activations are also possible.

The current observations indicate that the purely parallel model of Fig. 10A cannot be correct. If ATP were to act independently through Ca2+, PGE2, and cAMP, blocking either the rise in intracellular Ca2+ or the formation of PGE2 should only partially inhibit the transport effects of ATP. This prediction is contrary to the observation that ATP-triggered shrinkage was completely blocked by either buffering intracellular Ca2+ levels or preventing PGE2 formation (Fig. 7, A and B).

In contrast, the series hypothesis (Fig. 12B) is consistent not only with the current results but also with a series cascade recently identified in MDCK cells (30). ATP-triggered elevation in cell Ca2+ activates phospholipase A2, stimulating cyclooxygenase-catalyzed PG synthesis and release. PGE2 occupancy of EP2 receptors is known to stimulate adenyl cyclase-mediated cAMP production specifically by bovine PE cells (2). In turn, cAMP activates Cl– channels (Fig. 10), permitting release of K+ and Cl– through parallel ionic channels and, secondarily, release of water (Figs. 6C, 7C, 8A, and 8C). The data of Fig. 8, B and C, obtained with the PKA-inhibitory analog of 8-BrcAMP, Rp-8-BrcAMPS, suggest that cAMP acts directly on the PE Cl– channel, not through activation of PKA. This concept is consistent with reports that Rp-cAMPS mimics cAMP-triggered activation of hyperpolarization-activated currents (3, 15) and Rp-cGMPS activates the photoreceptor (but not the olfactory) cyclic nucleotide-gated channel (19).

Potential physiological implications. The Cl– channels of PE and NPE cells differ in their unitary conductances (27, 37) and pharmacological profiles (28, 35). In principle, activation of the NPE Cl– channels facing the aqueous humor is expected to increase net secretion, whereas activation of PE channels facing the stroma is expected to reduce net secretion. Release of ATP from both NPE and PE cells (24) provides the basis for autocrine regulation of secretion. Ectoenzyme metabolism of ATP provides a source of adenosine, which activates NPE Cl– channels at ~3 μM concentration (5, 25). The results of the present work demonstrate that ATP itself activates PE Cl– channels at ~10 μM concentration (Figs. 1 and 3). Which effect predominates should depend on local purine concentrations, on ectoenzyme activities and receptor densities at both surfaces, and possibly on activities of additional modulators. In particular, occupancy of plasma membrane estrogen receptors is thought to trigger synergistic enhancement of ATP activation of PE cell Cl– channels (35). The basis for the synergism between the estrogen-triggered cascade and the Ca2+-PGE2-cAMP cascade triggered by ATP remains to be determined.

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REFERENCES


8. Civan MM, Coca-Prados M, and Peterson-Yantorno K. Pathways signaling the regulatory volume decrease of cultured


