Pathways for ATP release by bovine ciliary epithelial cells, the initial step in purinergic regulation of aqueous humor inflow

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Bafilomycin A1 acts by reducing the driving force for uptake of ATP from the cytosol into vesicles. The reducing agent dithiothreitol reduced probenecid-blockable ATP release. Similar results were obtained with NPE and PE cell lines. Panxennin PX1–3, connexins CX43 and CX40, and P2RXy were identified in native cells and cell lines by RT-PCR. PX1 mRNA expression was confirmed by Northern blots; its quantitative expression was comparable to that of CX43 by lines by RT-PCR. PX1 mRNA expression was confirmed by Northern blots; its quantitative expression was comparable to that of CX43 by RT-PCR. PX1 mRNA expression was confirmed by Northern blots; its quantitative expression was comparable to that of CX43 by RT-PCR. PX1 mRNA expression was confirmed by Northern blots; its quantitative expression was comparable to that of CX43 by RT-PCR. PX1 mRNA expression was confirmed by Northern blots; its quantitative expression was comparable to that of CX43 by RT-PCR. 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PX1 mRNA expression was confirmed by Northern blots; its quantitative expression was comparable to that of CX43 by RT-PCR. PX1 mRNA expression was confirmed by Northern blots; its qualitative expression was comparable to that of CX43 by real-time PCR. Heterologous expression of bovine PX1 in HEK293T cells enhanced swelling-activated ATP release, inhibitable by probenecid (28), and by an inhibitor of vesicular release (bafilomycin A1). We conclude that PX2y-independent PX1 hemichannels, pannexin-1 mediated ATP release from bovine ciliary epithelial cells.

pennxin-1; connexins; hemichannels; P2Xy ATP receptors; vesicular release

THE HYDROSTATIC PRESSURE WITHIN the eye, the intraocular pressure (IOP), is directly dependent on the rate of formation of aqueous humor by the ciliary epithelium and on the resistance to its outflow through the trabecular exit pathway. Reducing IOP is the only effective strategy documented to delay the onset and slow the progression of glaucomatous blindness (1, 12, 13, 22). Blocking transport components to reduce the rate of aqueous humor formation is a current approach for treating glaucoma.

The ciliary epithelium transfers solute, largely Na+ and Cl−, from the supporting stroma sequentially through the pigmented (PE) ciliary epithelial cells, gap junctions, and nonpigmented (NPE) ciliary epithelial cells to the aqueous humor (Fig. 1). Na+ and Cl− are taken up at the stromal surface largely by electroneutral transporters and released into the aqueous humor through Na+-K+–activated ATPase and Cl− channels, respectively (9, 37). The result of this osmotic gradient is generally thought to drive water from stroma to aqueous humor, although alternative mechanisms have been proposed (9, 37).

Chloride-channel activity at the stromal and aqueous humor surfaces of the ciliary epithelium may be of particular importance in modulating inflow (15). Stimulation of NPE Cl− channels at the aqueous humor surface is expected to increase inflow and IOP, whereas stimulation of PE Cl− channels at the contralateral surface should have the opposite effect, reducing net fluid inflow and IOP (Fig. 1). Agonists of A3 adenosine receptors (A3AR) stimulate NPE-cell Cl− channels (7, 8) and trigger fluid release from isolated NPE cells (27). Consistent with the in vitro data, adenosine stimulates aqueous humor secretion in rabbits independent of ciliary blood flow (23). Furthermore, agonists selective to A3ARs increase, whereas A3AR antagonists reduce mouse IOP (2). As predicted, A3AR-null mice display reduced IOP (3).

At the contralateral ciliary epithelial surface, ATP increases intracellular cAMP production, directly activating PE-cell maxi-C1− channels (17, 19). The Cl−–channel stimulation produced by ATP is enhanced by tamoxifen (28). The physiological significance of the PE-cell Cl−–channel stimulation on modulating net inflow has not yet been established.

The initial and enabling step of purinergic regulation of Cl− channels at the two surfaces of the ciliary epithelium is the release of ATP by the PE and NPE cells (26). The ATP acts directly on P2Y2 and possibly other ATP receptors of the PE cells (34) and is metabolized to adenosine by ectoenzymes to activate the A3ARs of the NPE cells (26). Despite the importance of the ATP release step in initiating purinergic regulation of inflow, the underlying mechanisms have been unknown. Multiple release pathways have been thought operative in other cells (4, 30, 33, 36). In the present study, we have found evidence that the recently identified pannexin-1 (PX1) and connexin (Cx) wide-bore hemichannels and vesicular release all participate in ATP release by NPE and PE cells.

MATERIALS AND METHODS

Cellular model. Mixed bovine pigmented and nonpigmented ciliary epithelial (bCE) cells were isolated and studied in primary culture as previously described (17). Transformed normal bovine pigmented (bPE) and nonpigmented (bNPE) ciliary epithelial cell lines (provided by Dr. M. Coca-Prados, Yale University), and HEK293T cells (ATCC, Manassas, VA) were maintained in DMEM high-glucose medium containing 10% fetal bovine serum, 2 mM L-glutamine, and 50 μg/ml of gentamicin at 37°C in a humidified atmosphere of 5% CO2 and...
Purinergic regulation of aqueous humor formation. Fluid is secreted from the stroma through the bilayered ciliary epithelium into the aqueous humor. ATP release by pigmented ciliary epithelial (PE) cells leads to a cAMP-mediated, tamoxifen (TMX)-enhanced activation of maxi-CI− channels (17, 19, 28), subserving Cl− recycling that would reduce net secretion. Ectoenzymatic degradation of ATP released by the nonpigmented ciliary epithelial (NPE) cells produces adenosine (ADO) that can activate A3 adenosine receptors, thereby stimulating Cl− channels and fluid secretion (7, 8, 27).

95% air. Cells were 1/10 subcultivated upon reaching 90% confluence and were studied from passages 10–30.

Solutions and pharmacological reagents. The isotonic solution (295–305 mosmol/kg) contained (in mM) 110 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgCl2, 30 NaHCO3, 1.2 KH2PO4, 15 HEPES, and 10 glucose. Selectively omitting NaCl reduced osmolality to 100 mosmol/kg (67% hypotonicity). Intermediate osmolalities were generated by appropriate mixing of the iso- and hypotonic solutions. In some experiments, the extracellular free Ca2+ ([Ca2+]o) was reduced to 0.1 mM with 5.1 mM CaCl2 and 5 mM EGTA. The final osmolalities were verified and pH values were adjusted to 7.4 before each experiment. Biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO), except for probenecid (PRO; Alfa Aesar, War Hill, MA) and dithiothreitol (DTT; Fisher Scientific, Pittsburgh, PA). Chemicals for cell culture were obtained from Gibco Invitrogen.

DMSO (<0.5%) was used to solubilize hydrophobic drugs, with controls exposed to the same concentration. Unless otherwise noted, all the experiments were done at 25°C.

ATP measurement. ATP release was measured by the luciferin-luciferase reaction with light emission recorded with a microplate luminometer (Synergy 2, BioTEK, Winooski, VT) (31). Cells were trypsinized and plated onto 96-well microplates (Corning Costar, Corning, NY) at 0.1 million cells per well, leading to confluence within 1–3 days. Mixed BCE cells were seeded directly after harvest, and these plates were studied within 2 days to minimize loss of NPE cells during culture. Uncontrolled mechanosensitive ATP release was minimized by removing the culture media of the bath and preincubating the cells for 1 h with 100 μl of isotonic solutions with or without drugs. Thereafter, 75 μl of isotonic solution was replaced with an equal volume of test solution to establish the final osmolalities and drug concentrations. The plate was placed in the microplate luminometer 30–60 s after addition of the test solution, and 10 μl of the ATP assay solution was injected into each well through the internal injector system. Measurements were started immediately after the finish of the dispensing (i.e., 60–90 s after adding the test solution) and taken at 2-min intervals for each well for 2 h, using an integration time of 0.2 s/measurement. The ATP assay solution was prepared from one vial of the ATP assay mix (FL-AAM, Sigma) diluted in 5 ml distilled water and 5 ml ATP assay mix dilution buffer (FL-AAB) following the manufacturer’s preparation instructions. ATP concentrations were calculated at each time point from a standard curve mapping arbitrary light units to ATP concentrations. Separate standard curves were used in experiments involving substances at concentration high enough to interfere with the ATP assay [e.g., addition of flufenamic acid or 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) or changing ionic strength]. Inhibition of ATP release was calculated from Eq. 1.

\[
\text{Inhibition (\%)} = 100\% \cdot \frac{(C_{\text{max}} - C)}{(C_{\text{max}} - C_{\text{con}})} \tag{1}
\]

\[C_{\text{max}}\] was the maximal ATP concentration (C) after hypotonic treatment, and \[C_{\text{con}}\] was the control value of the ATP concentration in the isotonic bath at the same time point. The peak time of each well was recorded as the time point following the ATP release reached its maximum, which appeared generally within the first to third measurement point following hypotonic challenge, i.e., 0–6 min after starting to record data.

The reducing agent DTT was one of the inhibitors of PX1 activity utilized in the present study. However, DTT has also been used to stabilize luciferase activity against oxidative stress. In principle, this stabilizing action might have confounded quantitative analysis of the response to DTT, leading to underestimation of the inhibition. However, the ATP assay mix and ATP assay mix dilution buffer we purchased from Sigma contain DTT to stabilize the enzyme, with a calculated final concentration of ~10 μM in each well. This small concentration apparently optimized luciferase stabilization because the time course of luciferase activity over 2 h was identical in solutions containing the highest measured concentration of released ATP (150 nM) with (N = 39 wells) or without (N = 38 wells) 10 mM DTT (P > 0.3). Insofar as the measured peak ATP used in our analyses appeared within the first 6 min after hypotonic challenge, the potential underestimation of DTT’s inhibitory effect was negligible.

Cell viability assays. Release of lactate dehydrogenase (LDH) from damaged cells was assayed colorimetrically to monitor viability. Aliquots of cells were similarly challenged with the stimuli specified, and these plates were studied within 2 days to minimize loss of NPE cells during culture. Uncontrolled mechanosensitive ATP release was measured by the luciferin-luciferase reaction with light emission recorded with a microplate luminometer (Synergy 2, BioTEK, Winooski, VT) (31).


definition of Cell Viability (%).

\[
\text{Cell Viability (\%)} = \frac{100\% \cdot \left(OD_{\text{high-ctrl}} - OD_{\text{sample}}\right)}{OD_{\text{high-ctrl}} - OD_{\text{low-ctrl}}} \tag{2}
\]

RT-PCR. Total RNA isolated from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) was digested with RNase-free DNase I to avoid possible genomic DNA contamination and was then reverse transcribed into cDNA by use of TaqMan Reverse Transcription Reagents [Applied Biosystems (ABI), Foster City, CA], according to the manufacturer’s instructions. Cell genomic DNA treated with RNase A and proteinase K was extracted by use of Purelink Genomic DNA Kits (Invitrogen, Carlsbad, CA). PCR was performed with the AccuPrime Taq DNA polymerase High Fidelity Kit (Invitrogen) under the recommended conditions. Primers used for gene-specific amplification are shown in Supplementary Table S1A (the online version of this article contains supplemental data). PCR products were separated on 1% agarose gels containing 0.05% ethidium bromide.
Bands were visualized under ultraviolet light, sized, and photographed by the Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA). The successfully amplified products were recovered by gel extraction and further verified by sequencing in the DNA Sequencing Facility of the University of Pennsylvania.

**Northern blotting.** Cell mRNA was isolated and concentrated from total RNA with Oligotex mRNA Mini Kit (Qiagen) by following the supplier’s protocol, with 1.5 μg loaded for each lane in agarose gels and electrophoresed in the NorthernMax-Gly System (ABI). Separated mRNA was then transferred to BrightStar-Plus membranes (ABI) and cross-linked by ultraviolet light. Templates for generating biotin-labeled probes were obtained by routine PCR amplification using gene-specific primers listed in Supplementary Table S1A. Products were randomly labeled using the BioPrime DNA Labeling System (Invitrogen), followed by column purification (Purenlink PCR Purification System, Invitrogen). Biotinylated DNA probes were diluted to a working concentration of 10 pM. Membranes were initially prehybridized in ULTRAhyb Buffer at 42°C for 2 h, and heat-denatured probes were then added to the blot for an overnight incubation at 42°C. After nonbinding probes were washed away, bands were visualized by treatment with BrightStar BioDetect Kit (ABI), by using BrightStar Biotinylated Millennium Marker (ABI) as the size indicator.

**Real-time quantitative PCR.** Cell cDNA templates were obtained as described above. The TaqMan gene expression assay was performed at least in quadruplicate for each cDNA sample. Assays were run with Fast Universal TaqMan Master Mix on the Applied Biosystems Prism 7500 Fast Sequence Detection System by using the fast ramping protocol. FAM-labeled MGB TaqMan probes utilized in the assays are listed in Supplementary Table S1B. The expression levels of indicated genes were all normalized to that of Cx43 in native bCE cells after 2−ΔΔCt calculation, with bovine peptidyl proline isomerase B as the endogenous control.

**Plasmid preparation and transfection of HEK293T cells.** Reverse-transcribed cDNA from primary bCE cells was used for PCR amplification. Bovine PX1 (bPX1) was initially propagated with the entire coding sequence (CDS) of PX1 primer pair (Supplementary Table S1A), and amplified products were ligated into pGEM T-easy (Promega, Madison, WI). The CDS of the long isoform of PX1, identified and used in the ensuing experiments, was further amplified from pGEM-bPX1 by PCR with primers 5’-GTGGATATTCGCCACCCCCTCCCAGAC-3’ (sense) and 5’-ATGAGCATCCCAGCAAGACTGTGACAGAAGTC-3’ (antisense), with restrictive enzyme digestion sites added, and with deletion of the stop codon. The expected product was inserted into pEGFP-N1 (Clontech, Takara) following an EcoRI/BamHI double digestion. The other mammalian cell-expressing plasmid, bPX1-myc-6×His, was similarly constructed by transfecting the CDS of bPX1 from bPX1-EGFP to pcDNA3.1-myc-6×His with direct HindIII/BamHI digestion and ligation (Fast Ligation Kit, Roche). All constructs were confirmed by full DNA sequencing. HEK293T cells were seeded 24 h prior to experimentation and reached 80% confluence on the day of transfection. Plasmids (5 μg) were delivered into the cells by Lipofectamine 2000 (Invitrogen). All constructs were confirmed by full DNA sequencing.

**Total proteins were extracted from HEK293T cells.** The HEK293T cells were plated on coverslips and transfected by the same protocol mentioned above to optimize transfection efficiency as well as maximize protein expression. Because of the initial high-density seeding and the vigorous cellular propagation, the HEK293T cells were always overcrowded 48 h after treatment and readily detached from coverslips during the immunostaining procedure. Furthermore, transfected and nontransfected cells were intermingled, obscuring the definition of the plasma membranes. To enhance the contours of the individual cells, we followed and extended the method of Ma et al. (24). Cells were trypsinized and reseeded on poly-L-lysine-coated coverslips 48 h posttransfection and were permitted to attach firmly to the underlying surface over 4 h before fixation. Because of the relatively short time period between seeding and fixation, the cells appeared round, rather than polygonal. The HEK293T cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 100 μM digitonin for 10 min, followed by blocking with 1% BSA-PBS for 1 h. Diluted primary antibodies were probed with samples in a humidified chamber overnight at 4°C. Coverslips were rinsed in PBS three times and then incubated with fluorophore-conjugated secondary antibodies for 1 h. DAPI (0.15 μg/ml) was added to counterstain the nuclei fluorescently. Coverslips mounted with fluorescent mounting medium (DAKO, Carpinteria, CA) were observed by confocal laser scanning microscopy (Leica TCS SP5 AOB spectral confocal microscope, Leica Microsystems). Single layers of 0.4-μm thickness were photographed. The antibodies used for detection were: mouse anti-myc (1/500), mouse anti-6×His (1/500), rabbit anti-mouse IgG-Alexa Fluor 488 (1/1,000), and rabbit anti-mouse IgG-Alexa Fluor 555 (1/1,000), all from Invitrogen.

**Statistics.** Student’s t-test, paired or unpaired as appropriate, was applied in comparing two sets of data, and one-way ANOVA was applied to compare three or more sets of data. Statistical analyses were performed with SigmaStat (Aspire Software International). Unless otherwise stated, the results are presented as means ± SE, and n is the number of wells studied. A probability (P) less than 0.05 was considered statistically significant.

**RESULTS**

**ATP release from ciliary epithelial cells.** The ATP concentration in control isotonic bath solutions was 6.1 ± 0.3 nM (mean ± SE, n = 978 wells), 15.7 ± 0.7 nM (n = 670), and 13.8 ± 0.4 nM (n = 694) after incubation of bCE, bPE, and bNPE cells, respectively. This low baseline level was stable during more than 2 h of measurement. Hypotonicity (50%) triggered rapid release of ATP from bCE cells, increasing bath concentration by 5.5-fold to 39.4 ± 2.3 nM (n = 530, P < 0.001), with a time to peak response of 0.8 ± 0.1 min. Similarly, hypotonicity increased bath concentrations of ATP by 9.2 ± 0.3-fold in bPE cells (n = 646) and by 7.7 ± 0.2-fold in bNPE cells (n = 660).

Measured by LDH release (Eq. 2), cell viability was close to 100% after 4 h of incubation in control isosmotic solution or 50% hypotonicity, at external Ca2+ concentrations of 2.5 or 0.1 mM (Supplementary Fig. S1). The ≥99% viability of cells following incubation suggested, albeit did not prove, that ATP release during those periods of measurement were mediated by
physiological pathways, whose pharmacological profiles were next determined.

**Mechanisms supporting swelling-activated ATP release from native bCE cells.** To identify the mechanisms mediating ATP release, 11 inhibitors were utilized in first probing ATP release from 4,178 drug-treated and 3,807 control wells of bCE, bPE, and bNPE cells (Fig. 2, Supplementary Tables S2–S3). The most selective of these blockers in distinguishing between the recently identified PX1 and longer-recognized Cx hemichannels is PRO. Although PRO exerts effects on other targets, it inhibits PX1, and not Cx, hemichannels (24, 35). At 0.5 and 1.0 mM, PRO inhibited hypotonicity-induced ATP release from bCE cells by 23 ± 4 and 47 ± 2% (Fig. 2, Supplementary Table S2). Mefloquine (MFQ) has also been found to be a selective inhibitor of PX hemichannels at low concentrations (10–100 nM) by some (20) but not by all investigators (24). In the present study, MFQ (100 nM) inhibited hypotonicity-induced ATP release from bCE cells by 23 ± 6% cells (Fig. 2). A third relatively selective inhibitor of PX1 hemichannels is carbenoxolone (CBX), with a reported IC50 value of ~5 μM against PX1, 5- to 20-fold lower than that needed to inhibit Cx hemichannels (5a, 24). In the present study, CBX concentrations of 1, 3, and 30 μM inhibited hypotonicity-induced ATP release by 33 ± 4, 40 ± 3, and 58 ± 3% (Fig. 2, Supplementary Table S2), respectively, corresponding to an IC50 of 1.6 ± 0.3 μM. The reducing agent DTT (10 mM) also inhibited swelling-activated release from bCE cells by 29 ± 3% (Fig. 2), consistent with its known partial inhibition of PX1 channels (6). Application of DTT together with 1 mM PRO did not enhance the inhibition produced by 1 mM PRO, alone (P > 0.05) (Fig. 2). The results obtained with PRO and CBX suggest that ~40% of the ATP release triggered by swelling native bovine CE cells might be mediated by PX hemichannels.

The relatively specific blockers of Cx hemichannels, heptanol (HEP, 1 mM) (24), and flufenamic acid (FFA, 30 μM) (5a), inhibited swelling-activated ATP release from bCE cells by 49 ± 4 and 50 ± 4%, respectively (Fig. 2). As expected from the known inhibitory effects of [Ca2+]ext on Cx hemichannel activity (5a), reducing [Ca2+]ext from 2.5 to 0.1 mM increased both swelling-activated ATP release (P < 0.001) and the efficacy of 1 mM HEP in inhibiting that release (P < 0.005) (Supplementary Table S2). Gadolinium (Gd3+, 50 μM), a blocker of both Cx hemichannels and maxi-anion channels (36), reduced hypotonically evoked ATP release by 30 ± 5% (Fig. 2); however, the inhibition from combined application of Gd3+ and 1 mM HEP (56 ± 1%) had no greater effect than HEP alone (P > 0.05). We also used three approaches to block PX and Cx hemichannels simultaneously. Combining 30 μM CBX with 30 μM FFA, applying 1 mM PRO together with 1 mM HEP, or using a high concentration (100 μM) of the nonselective channel blocker NPPB raised the inhibition of hypotonicity-triggered ATP release to 74 ± 5, 84 ± 1, and 75 ± 3%, respectively. Thus the putative block of both PX and Cx hemichannels inhibited swelling-evoked ATP release by ~80%. These results, taken together with the above estimated contribution by PX hemichannels of ~40%, suggest that Cx hemichannels might provide conduits for ~40% of the swelling-triggered ATP release.

Neither the antagonist KN-62 (1 μM) of P2RX7 nor the blocker phloretin (PHL, 30 μM) of voltage-sensitive outwardly rectifying (VSOR) anion channels had an effect on hypotonicity-activated ATP release. However, bafilomycin A1 (BAF, 2 μM), an inhibitor of vesicular transportation, lowered the release by 25 ± 2%, suggesting that exocytosis also contributed to swelling-stimulated release. BAF acts by reducing the driving force for ATP uptake from the cytosol into vesicles. Simultaneous application of 1 mM PRO, 1 mM HEP, and 2 μM BAF to block PX hemichannels, Cx hemichannels, and exocytosis, respectively, inhibited swelling-activated ATP release by 91.8 ± 2.3% (Fig. 2). The nearly complete inhibition produced by blocking the major presumed pathways of hypotonicity-triggered ATP release is consistent with the measurements of LDH release, which indicated no loss of ciliary epithelial cell viability under the hypotonic conditions employed.

**Comparison of mechanisms supporting ATP release from native bCE cells and from ciliary epithelial cell lines.** The results obtained with the bPE and bNPE cell lines were very similar to those measured with the native bovine cells, with slightly different potencies and efficacies (Supplementary Table S3). The closeness of the similarity is illustrated by Fig. 3.
We addressed the possibility of genomic contamination by comparing bands for GAPDH mRNA generated by cDNA and by genomic DNA (Fig. 4). GAPDH primers (pair 1) designed according to exon 5 and exon 11 produced a single small band at 606 bp by using cDNA as the template, whereas no band was visible when reverse-transcriptase was omitted. A larger band of 1,251 bp was visible only when PCR templates were cell genomic DNA, rather than cDNA reverse-transcribed from total RNA after DNase I treatment. These control results eliminate the possibility of genomic DNA contamination, substantiating that the bPX1 we acquired was undoubtedly from mRNA.

To document the expression of bPX1 further, Northern blots were also performed, demonstrating the physiological transcription of bPX1 into mRNA products in bCE, bPE, and bNPE cells (Fig. 5, B and C).

The relative expression levels of the genes generating ATP-release pathways were quantified by real-time quantitative PCR. Cx43 was in highest abundance among the functional ATP-releasing channels in bCE cells. Copies of PX1-L were expressed ~40% as highly as of those of Cx43, with the total copies of PX1 (PX1-L and -S) approximately equal to those of Cx43. Consistent with our previous measurements (42), the expression of Cx40 mRNA was ~0.3% that of Cx43. The relative gene expression of P2RX7 was approximately twice that of Cx40 in bCE cells. Expression levels of P2RX7 and Cx43 were largely similar in bPE and bNPE cells. In both cell lines, the copy numbers of Cx43 mRNA were less and of P2RX7 mRNA were more than those of bCE cells (Fig. 6).

**Heterologous expression of bPX1 in HEK293T cells.** Despite our past success in Cx43 knockdown in bCE cells (42), bCE, bPE, and bNPE cells proved resistant to overexpression of bPX1–3 and P2RX7 in mRNA from the 3 cell preparations by RT-PCR. The results obtained with GAPDH (primer pair 1) confirmed that the cDNA derived from total RNA is free of genomic DNA (gDNA) contamination, because the expected products (606 bp) are observed only in the presence of reverse transcriptase [RTase (+)], distinct from those (1,251 bp) generated by gDNA.

![Fig. 3. Correlation between inhibition of ATP release from native cells (bCE) and cell lines (bPE and bNPE). Data plotted reflected measurements after exposure to 1 mM PRO, 10 mM DTT, 100 nM MFQ, 1 mM HEP, 30 μM CBX, 2 μM BAF, 50 μM Gd⁴⁺, 1 μM KN-62, 30 μM NPPB, or 1 mM PRO + 1 mM HEP. The line is generated from the linear regression relating inhibition of bPE and bNPE cell lines (x) to inhibition of bCE cells (y) by the same drug at the same concentration: y = (12.15 ± 6.84) + (0.83 ± 0.15)x, with a correlation coefficient (R) of 0.78 (P < 0.0001).](http://ajpcell.physiology.org/)

**Molecular identification of ATP-permeable conduits in bovine ciliary epithelial cells.** Cx43 is expressed at least a hundredfold more than Cx40 in bCE cells (42). No information has been previously reported concerning expression of P2RX7 or PX1 in these cells. We documented the expression of bPX2, bPX3, and bP2RX7 in bCE, bPE, and bNPE cells with RT-PCR and subsequent DNA sequencing (Fig. 4).

Before the present study, GenBank had no documentation for the sequence of bPX1. In keeping with the sequence predicted by computational analysis with the name “Similar to Pannexin-1,” three pairs of primers were synthesized and used experimentally to detect possible bPX1. All of the short fragments were successfully amplified with expected sizes (Figs. 4 and 5A). With the primers designed specifically from 5'- and 3'-untranslated regions, two bands were obtained, a short amplicon (PX1-S) and a long form (PX1-L) of the PX1 CDS (Fig. 5A). The results of DNA sequencing and subsequent alignments with PX1 of other species verified that PX1-L contains the whole CDS of bPX1, sharing >80% protein identity with that of human, whereas PX1-S is 140 bp shorter in lacking exon 2 (Supplementary Figs. S2–S3). This omission must result in a reading-frame shift and, if translated, expression of a different protein. The CDS of bPX1 mRNA (PX1-L) has been deposited into GenBank for the first time (approved accession number: HM036582; GI number: 296398829). The bovine PX1-L we have cloned is 82% identical with human PX1 and shares 77–79% identity with PX1 of the rat, mouse, and dog (Supplementary Fig. S3).
and small interfering RNA knockdown of PX1, even when transfection was extended to cytotoxicity. To test whether the bPX1 protein could be overexpressed in other cells, we transfected HEK293T cells with either bPX1-EGFP or bPX1-myc-6×His plasmids. Both transfections succeeded in transiently expressing tagged bPX1, confirmed by Western blotting (Fig. 7A, Supplementary Fig. S4B). However, immunocytochemistry revealed that the large GFP tag interfered with trafficking of bPX1 to the plasma membranes (Supplementary Fig. S4A).

Identical results have been observed in HEK293 cells transfected with either human or mouse PX1 with GFP or DsRed tags (24). In contrast, trafficking of the bPX1 fusion protein was not disturbed by the small myc/6×His tag. Single-layer confocal microscopic figures demonstrated that bPX1 proteins were localized almost exclusively at or near the plasma membranes (Fig. 7B).

We next tested whether heterologously expressed bPX1 protein was functional in HEK293T cells. Our pharmacological results (see above) had suggested that PX1 is a major conduit for hypotonicity-induced ATP release in the bovine ciliary epithelial cells, as in a number of other cells. Hypotonicity (50%) increased ATP release over baseline by 4.4 ± 0.1-fold (n = 197) in HEK293T cells transfected with blank vector. Overexpression of bPX1 did not change baseline ATP release but increased the hypotonicity-triggered ATP release from 4.4 to 6.2 ± 0.2-fold (n = 171, P < 0.001 compared with the mock cells). Correspondingly, bPX1 overexpression increased efficacy of inhibition by 1 mM PRO to 42 ± 2% (P < 0.001). In control cells, the inhibition of swelling-activated ATP release by 1 mM PRO was 30 ± 2%, consistent with the known expression of human PX1 in HEK293 cells (24). The results demonstrate that bPX1 can be functionally overexpressed, supporting the conclusion based on pharmacological data that bPX1 is a major release mechanism for hypotonicity-triggered ATP release by bovine ciliary epithelial cells.

**DISCUSSION**

The present work focuses on ATP release, the enabling step in purinergic regulation of aqueous humor formation. In our initial study, we found that swelling of continuous lines of bovine NPE and PE cells triggered ATP release through NPPB-inhibitable pathways (26). At that time, little was known about the possible molecular identities of the release mechanisms. Hemichannels had not yet been considered a conduit for ATP release.

**Molecular basis for ATP release.** The present results document gene expression of PX1, Cx43, and P2RX7 in native bovine ciliary epithelial cells and in lines of bovine NPE and PE cells. Cx40 is expressed two to three orders of magnitude less than Cx43. PX and Cx hemichannels and P2RX7 have been found to release ATP in other cells. Insofar as earlier efforts to identify the bovine form of PX1 (bPX1) led only to a pseudogene, we verified that heterologous expression of the long isoform of bPX1 in HEK293T cells enhanced swelling-activated ATP release. The long isoform of bPX1 displays high identity (77–82%) with PX1 from several mammalian species, including the human isoform, and is expressed to a comparable extent as Cx43. The other members of the PX family, PX2 and PX3, were also identified by RT-PCR. PX2 is known to form heteromeric hemichannels with PX1, thereby reducing the channel conductance (5a). PX3 has not been found to form functional hemichannels but has recently been observed to promote differentiation in chondrocytes (21).

Several other putative channels for ATP release have been excluded by previous study of gene expression and by functional measurements. First, the cystic fibrosis transmembrane conductance regulator (CFTR) is not functionally present in the bovine PE and NPE cell lines because cAMP does not stimu-

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**Fig. 5.** Long (PX1-L) and short (PX1-S) isoforms of the complete coding sequences of PX1 (PX1 CDS) in the 3 cell preparations, verified by RT-PCR (A). Biotinylated probes generated by random labeling of products amplified with gene-specific PCR primers, pair 2 for GAPDH and pair 3 for PX1, were used in later detection of GAPDH (B) and PX1 (C) by Northern blotting.

**Fig. 6.** Relative gene expression in the 3 cell preparations measured by real-time PCR.
late ATP release, and the CFTR inhibitors glibenclamide and
diphenylamine-2-carboxylate do not reduce swelling-activated
ATP release (26). Furthermore, Northern blot analysis did not
detect mRNA for CFTR in a continuous line of human NPE
cells (10). These in vitro data are consistent with the observa-
tion that aqueous humor flow is unaltered in patients with
cystic fibrosis who suffer loss of function of CFTR (25).

Second, the conduit for large organic osmolytes, known both as
VSOAC (38) and VSOR (14), is excluded because the inhibitor
tamoxifen was found not to affect swelling-activated ATP
release by the bovine PE and NPE cell lines (26), and the
inhibitor PHL had no effect on swelling-activated ATP release
in the present study. Third, the ineffectiveness of both tamox-
ifen and verapamil on hypotonicity-triggered ATP release
excludes P-glycoprotein as a possible release mechanism (26).

Functional contributions of PX1 and Cx hemichannels and
exocytosis to swelling-activated ATP release. The present
study establishes that PX1 and Cx hemichannels and vesicular
release constitute the major swelling-activated release path-
ways in native bovine ciliary epithelial cells and in lines of

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Fig. 7. Heterologous expression of bPX1 in
HEK293T cells. Western immunoblots confirm ex-
pression of bPX1 tagged with myc-6×His, using
anti-myc and anti-6×His antibodies (A). Confocal
images demonstrate the localization of the PX1
close to or within the plasma membrane (B).
bovine NPE and PE cells. Of the 11 inhibitors used to distinguish the release pathways, PRO, HEP, BAF, and KN-62 proved to be the most informative probes. The results obtained with these inhibitors permit estimation of the relative contributions of the release pathways. Simultaneously blocking PX1 hemichannels, Cx hemichannels, and vesicular ATP release with PRO, HEP, and BAF, respectively, inhibited swelling-activated ATP release by 92 ± 2%; therefore, putative cell damage could only have played a negligible role. Because of possible cross-target inhibitions, quantitative estimations of the relative importance of the outflow pathways are necessarily approximate. However, the following considerations suggest that the current estimations drawn from ~8,000 measurements of ATP release in control and experimental wells are likely realistic.

PRO is the only hemichannel blocker considered to inhibit PX1 without known cross-inhibition of Cx hemichannels (24, 35). Silverman et al. (35) estimated the IC$_{50}$ of PRO to be 150 µM but needed 1 mM to abolish the currents through PX1 channels heterologously expressed in *Xenopus* oocytes. Ma et al. (24) have noted differences in pharmacological profiles following expression of P2RX7 together with PX1 in mammalian cells and measured an IC$_{50}$ of 350 µM for PRO targeting PX1 hemichannels overexpressed in mammalian cells. On the basis of these published reports, the 47 ± 2% inhibition produced by 1 mM PRO sets an upper bound to the percentage contribution by PX1 channels in the native bovine ciliary epithelial cells. The percentage inhibition by DTT of 29 ± 3% provides a lower bound to the percentage contribution of PX1 hemichannels because DTT has been reported to inhibit PX1 only partially (6). Our observation that the simultaneous application of DTT and PRO did not enhance the inhibition by PRO alone supports the idea that DTT was selectively acting on PX1 hemichannels. These considerations indicate that PX1 channels contribute 29–47% of the total swelling-activated ATP release.

HEP is a blocker of Cx hemichannels that does not appear to inhibit PX1 at 0.5 mM but does cause inhibition at 3 mM (29). In the present study, 1 mM HEP appeared to be relatively selective for Cx over PX1 hemichannels because 1 mM HEP and 1 mM PRO were approximately additive in blocking swelling-activated ATP release (Fig. 2, Supplementary Table S3). BAF interferes with vesicular uptake of ATP within the cell and is thereby commonly used to inhibit vesicular ATP release to the extracellular space (11, 18, 40, 41). BAF also appeared to be relatively specific because it further increased the inhibition exerted simultaneously by PRO and HEP (Fig. 2, Supplementary Table S3).

These considerations indicate that our estimations of the relative contributions of the three major outflow pathways in these cells are likely well-founded approximations. The data suggest that hypotonicity-triggered ATP release proceeds ~40% through PX hemichannels, ~40% through Cx hemichannels, and the remainder through vesicular release.

**Potential inhibitions of upstream signaling cascades.** In addition to the considerations of the foregoing paragraph, the possibility must be addressed that inhibitors of the ATP release pathways may have also exerted effects on the signaling cascade triggered by hypotonicity, upstream of pathway activation. Several observations suggest that such possible effects were likely negligible. First, the blockers of PX and Cx hemichannels and of vesicular ATP release produced additive inhibitions. If the blockers acted on upstream cascade events independent of the release pathways, the combined effects would be expected to be less than additive, unless the putative non-specific effects of the blockers are posited to be specific for different cascades. Second, expression of bPX1 hemichannels in HEK293T cells increased the percentage efficacy of PRO. If PRO were acting on upstream events independent of the release pathways, we would have expected no change in its efficacy. Third, we have previously found that blocking Cx hemichannels with HEP, even at 3 mM concentration, had no effect on the swelling of human NPE cells produced by 50% hypotonicity and exerted only a small effect on the regulatory volume decrease (43). Relative to their baseline isotonic values, the cell volumes of the HEP-treated and control NPE cells in that study were 148 ± 4% (N = 4) and 150 ± 3% (N = 4), respectively (P > 0.7), at the time of peak swelling 4 min after hypotonic challenge. Similarly, 30 min after applying hypotonicity, the volumes of the HEP-treated and control NPE cells were nearly identical (P > 0.9) at 127 ± 5 and 127 ± 2%, respectively. Thus, although we cannot entirely exclude the possibility that inhibitors of the release pathways may have also exerted upstream effects, those actions are unlikely to have significantly affected swelling-activated ATP release.

**Physiological implications and regulation of ATP release pathways.** Epithelial cells must constantly reconcile housekeeping needs with transepithelial transport functions. A transient mismatch between uptake of fluid at the stromal surface and release at the contralateral surface could cause catastrophic swelling of the ciliary epithelium. Swelling-activated ATP release might mitigate that eventuality. Ectoenzymatic formation of adenosine from ATP can stimulate A3ARs of the NPE cells, thereby activating Cl$^-$ channels and enhancing secretion into the aqueous humor. ATP release at the stromal surface of the PE cells can stimulate maxi-Cl$^-$ channels that might enhance fluid release back into the stroma (Fig. 1). The overall effect of swelling of the bovine ciliary epithelium is known to increase net Cl$^-$ secretion in the direction of the aqueous humor (16). Precisely how swelling leads to activation of PX1 and Cx hemichannels and vesicular release is unknown. However, membrane stretch is reported to activate both PX1 (4) and Cx hemichannels (5), consistent with the very rapid release of ATP following hypotonic challenge.

The data indicate that ATP release by ciliary epithelial cells is a function of oxidation-reduction state, as well as of cell volume. The reducing agent DTT inhibited swelling-activated ATP release from the native cells, bPE, and bNPE cells by 29 ± 3, 44 ± 4, and 25 ± 4%, respectively. In each case, the simultaneous application of DTT and 1 mM PRO produced almost exactly the same inhibition as did PRO, alone (Fig. 2, Supplementary Table S3), consistent with an observed partial inhibition by DTT of PX1 channels overexpressed in oocytes (6). Interestingly, the stimulation by DTT of Cx43 hemichannels overexpressed in HeLa cells (32) was not displayed by the bovine ciliary epithelial cells. This may reflect modulation by protein partners of the hemichannels in the different cell types. For example, the Kvβ3 subunit of the K$^+$ channel interacts with PX1 and interferes with its response to reducing agents (6). Downregulating PX1 activity in response to reducing conditions might impart benefit to the ciliary epithelial cells in...
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retaining ATP to support cell functions when oxygen is in short supply.

Future directions. The salient conclusions of the present study are that 1) the ciliary epithelium recruits multiple ATP-release mechanisms in response to cell swelling; 2) PX1 and Cx hemichannels and vesicular release are the principal pathways for swelling-activated ATP release by ciliary epithelial cells; and 3) the oxidation-reduction state is a substantial regulator of PX1-mediated ATP release from these cells. The contributions of the ATP release mechanisms are not identical with those of trabecular meshwork cells in the aqueous-humor outflow pathway (A. Li et al., unpublished observations). This observation raises the possibility of reducing IOP by selectively inhibiting ciliary ATP release without downregulating ATP release by outflow pathway cells. This novel possibility is under investigation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES