Review

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Mechanisms of ATP Release, the Enabling Step in Purinergic Dynamics

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Key Words

Ciliary epithelium • Trabecular meshwork • Intraocular pressure • Pannexin-1 hemichannels • Connexin hemichannels • $P2X_7$ ATP receptors • Vesicular release • A_3 adenosine receptors • A_1 adenosine receptors • Actin cytoskeleton

Abstract

The only effective intervention to slow onset and progression of glaucomatous blindness is to lower intraocular pressure (IOP). Among other modulators, adenosine receptors (ARs) exert complex regulation of IOP. Agonists of A_3ARs in the ciliary epithelium activate Cl^- channels, favoring increased formation of aqueous humor and elevated IOP. In contrast, stimulating A_1ARs in the trabecular outflow pathway enhances release of matrix metalloproteinases (MMPs) from trabecular meshwork (TM) cells, reducing resistance to outflow of aqueous humor to lower IOP. These opposing actions are thought to be initiated by cellular release of ATP and its ectoenzymatic conversion to adenosine. This view is now supported by our identification of six ectoATPases in trabecular meshwork (TM) cells and by our observation that external ATP enhances TM-cell secretion of MMPs through ectoenzymatic formation of adenosine. ATP release is enhanced by cell swelling and stretch. Also, enhanced ATP release and downstream MMP secretion is one mediator of the action of actin depolymerization to reduce outflow resistance. Inflow and outflow cells share pannexin-1 and connexin hemichannel pathways for ATP release. However, vesicular release and $P2X_7$ release pathways were functionally limited to inflow and outflow cells, respectively, suggesting that blocking exocytosis might selectively inhibit inflow, lowering IOP.

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Introduction

Glaucoma is a major cause of irreversible blindness world-wide [1] that is usually associated with elevated intraocular hydrostatic pressure (IOP). The symbols used

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Table 1	List of Abbreviations
A_1 and A_3 ARs	A_1 and A_3 subtype adenosine receptors
AH	aqueous humor
AQP1 and AQP4	aquaporin-1 and -4 water channels
BAF	bafilomycin A1 (inhibitor of vesicular ATP release)
bCE	bovine ciliary epithelial cell
CD73	ecto-5'-nucleotidase
CE	ciliary epithelial cell
CFTR	cystic fibrosis transmembrane conductance regulator Cl^- channel
Cx	connexin hemichannel or gap junction
DTT	dithiothreitol (a reducing agent partially blocking PX1)
E-NPP1-3	ecto-nucleotide pyrophosphatases/phosphodiesterases 1-3
E-NTPD2, E-NTPD8	ecto-nucleoside triphosphate diphosphohydrolases 2 and 8
FGF	fibroblast growth factor
GM6001	non-selective inhibitor of metalloproteinase activity
HCE	immortalized human nonpigmented ciliary epithelial cell
HEK293T	human embryonic kidney 293 cell line containing the SV40 large T-antigen
HEP	heptanol (relatively selective Cx inhibitor at 1 mM)
$I_{Cl,swell}$	swelling-activated Cl^- current
IOP	intraocular pressure
KN-62	inhibitor of $P2X_7$
MMP	matrix metalloproteinase
NPE	nonpigmented ciliary epithelial cell or cell layer
$P2X_7$	$P2X_7$ subtype ATP receptor
PX1	pannexin-1 hemichannel
PE	pigmented ciliary epithelial cell or cell layer
PRO	probenecid (inhibitor of PX1 hemichannels)
Pxs	pannexins
RT-PCR	reverse-transcription polymerase chain reaction
TM	trabecular meshwork
TM5	transformed human trabecular meshwork cell line

in this paper are listed in Table 1. Reducing IOP is the only intervention documented to delay the onset and slow the progression of glaucomatous blindness, even if IOP is not abnormally high [2-7]. In principle, IOP can be lowered by: slowing the rate of formation of aqueous humor, reducing the resistance to outflow through the pressure-sensitive (conventional) pathway, or shunting some of the trabecular outflow to exit through the relatively pressure-insensitive uveoscleral pathway. Among other regulators of intraocular pressure [8], adenosine receptors exert a complex modulation of aqueous humor dynamics (Fig. 1). Adenosine delivery to the aqueous humor face of the ciliary epithelium activates Cl^- channels [9, 10], tending to enhance fluid inflow into the eye and increasing IOP [11]. Adenosine delivery to the trabecular meshwork (TM) cells of the trabecular outflow pathway can stimulate release of matrix metalloproteinases (MMPs) [12], reducing resistance to exit flow [13, 14], and thereby IOP [11, 13, 15, 16]. The integrative balance of these opposing effects on IOP through actions on inflow and outflow determines whether pressure will increase or decrease. As indicated in the cartoon of Fig. 1 and discussed below, the source of the adenosine delivered to both inflow [17] and outflow cells [18] is thought to be through autocrine/paracrine ATP release and

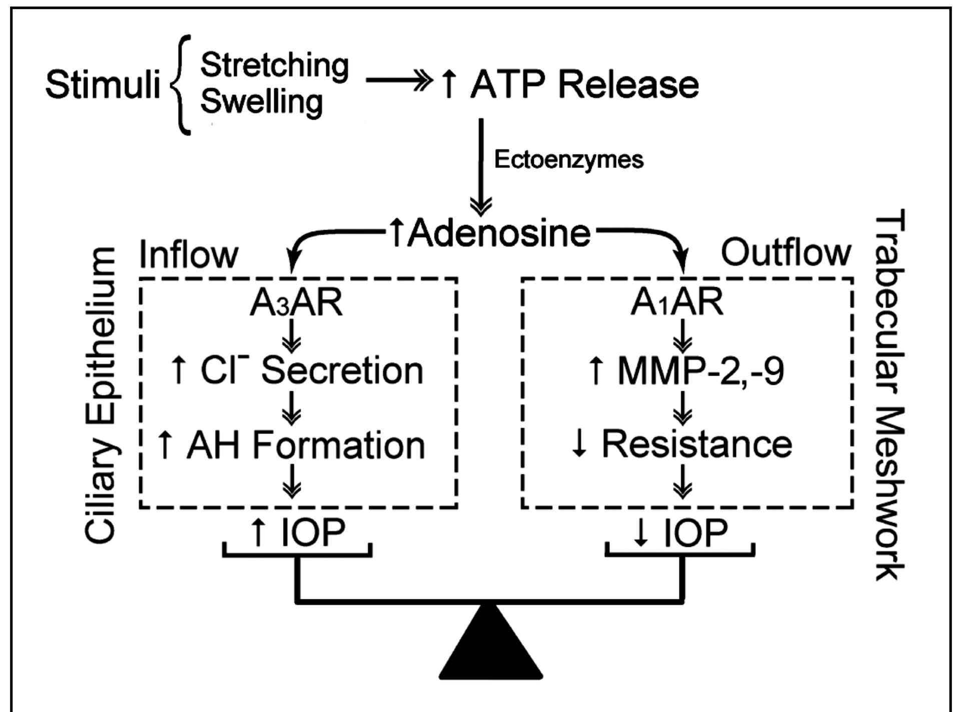

Fig. 1. Purinergic regulation of aqueous humor dynamics. Swelling or stretching of cells releases ATP, which can then be converted by ectoATPases to adenosine. Activation of A_3 adenosine receptors of NPE cells of the ciliary epithelium will activate Cl^- channels, favoring enhanced inflow and elevating IOP. In contrast, adenosine delivered to the trabecular meshwork cells of the outflow pathway activates A_1 adenosine receptors, stimulating secretion of matrix metalloproteinases-2 and -9, lowering outflow resistance and IOP.

subsequent metabolism by ectoATPases. If so, this raises the possibility that adenosine delivery to inflow and outflow tissues might be selectively modified pharmacologically, thereby permitting a novel strategy for lowering IOP. We have begun addressing this possibility in studies identifying the ATP release pathways and their regulation in inflow and outflow cells [18-20].

Formation of aqueous humor

The aqueous humor is formed by the bilayered ciliary epithelium comprising pigmented (PE) and nonpigmented (NPE) ciliary epithelial cell layers that cover the surface of the ciliary body. As with all secretory epithelia, fluid transfer depends on the net transfer of solute, here predominantly NaCl, with secondary passive transfer of water in response to the local osmotic gradient. Uptake of stromal Na^+ and Cl^- by the outer PE cell layer largely proceeds by electroneutral Na^+-K^+-$2Cl^-$ symports and paired Na^+/H^+ and Cl^-/HCO_3^- antiports. Thereafter, solute and water permeate gap junctions to the NPE cells, which release Cl^- through swelling-activated Cl^- channels and extrude Na^+ through Na^+,K^+-activated ATPase. AQP1 and AQP4 channels provide pathways for water release from NPE cells into the aqueous humor, but whether aquaporin channels play a role in uptake of water from stroma to PE cells is uncertain. The component plasma-membrane ion [21] and aquaporin [22] transporters subserving aqueous humor inflow are reviewed elsewhere.

Several considerations have suggested that NPE Cl^--channel activity limits the rate of aqueous humor secretion [23]. Selective agonists of A_3 subtype adenosine receptors (ARs) activate Cl^- currents [10] and cause cell shrinkage [9] of HCE immortalized human NPE cells [10]. The physiologic, non-selective A_3AR agonist adenosine also shrank the HCE cells under conditions where K^+ permeability was not rate-limiting. This shrinkage was abolished by preincubation with selective antagonists of A_3ARs [9]. The Cl^--channel target of A_3ARs shares macroscopic properties, and is likely identical, with swelling-activated Cl^- channels ($I_{Cl,swell}$) of NPE cells [10] facing the aqueous humor [24]. Despite considerable experimental effort, the molecular identity of the conduit subserving $I_{Cl,swell}$ is uncertain. Knockdown and blocking-antibody strategies have suggested that both ClC-3 [25-27] and pI_{Cln} [28] play roles in functional expression of $I_{Cl,swell}$ in NPE cells. As discussed elsewhere [21], whether these roles of ClC-3 [29, 30] and pI_{Cln} [31-33] are direct or indirect in ciliary epithelial and other cells remains unresolved.

Based on the foregoing observations with isolated cells, we expect that agonists and antagonists of A_3ARs will increase and decrease IOP, respectively. Measurements of IOP in the living mouse have confirmed

these predictions [11], and A_3-null mice display lowered IOP [34].

Outflow of aqueous humor

Outflow from the anterior chamber of the eye proceeds through two parallel pathways to eventually return to the venous system. The uveoscleral pathway begins with passage of aqueous humor between the ciliary muscle bundles, followed by permeation through several relatively pressure-insensitive routes [35]. Recent data suggest that uveoscleral exit may be more significant than previously believed. However, the parallel pressure-sensitive trabecular exit pathway is of particular interest since the elevated outflow resistance observed in glaucoma usually leads to an abnormally high IOP. The trabecular outflow pathway comprises, in series, the trabecular meshwork, juxtacanalicular tissue, Schlemm's canal and the collector channels. The site of, and mechanisms underlying, the resistance to trabecular outflow remain unclear [36]. Nonetheless, TM cells release metalloproteinases (MMPs) [12, 37], and increased MMP activity reduces outflow resistance of human [38] and bovine [14] preparations. One of adenosine's mechanisms of action in the outflow tract involves MMPs since agonists of A_1 subtype ARs stimulate secretion of both MMP-2 [12, 20, 39] and MMP-9 [20]. The MMPs secreted after agonist activation of A_1ARs are functional, reducing resistance within 20 min by 20-25%, an effect nearly completely abolished by the non-selective MMP inhibitor GM6001 [14]. The A_1AR-mediated reduction in outflow resistance leads to decreased IOP, measured in multiple species, including non-human primates [11, 13, 15].

Balance of Purinergic Effects on IOP

The foregoing observations indicate that the physiologic agonist adenosine exerts opposing effects on IOP through its actions on inflow and outflow (Fig. 1). Adenosine activates A_3ARs of NPE cells of the ciliary epithelium to increase Cl^- release, tending to enhance inflow and thereby raising IOP. In contrast, adenosine stimulates A_1ARs of the trabecular meshwork to enhance MMP-2 and MMP-9 secretion, reducing outflow resistance and thereby lowering IOP. In each case, the source of adenosine delivery to the ARs has been thought to be ATP release, with subsequent conversion of ATP to adenosine by ectoenzymes [17, 18].

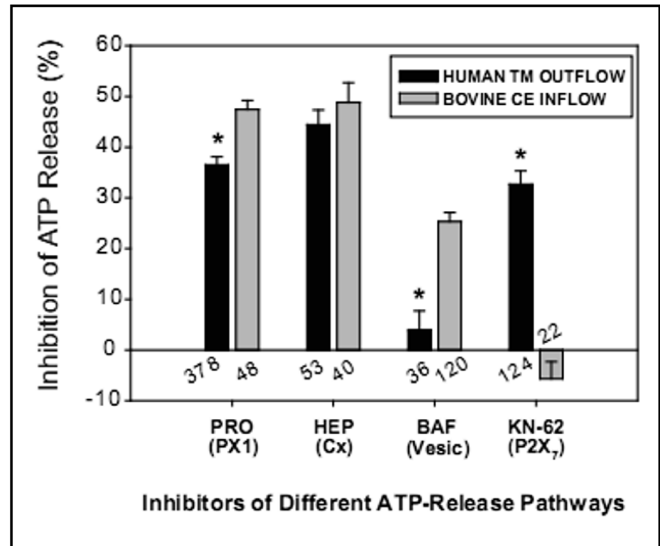

Fig. 2. Comparison of swelling-activated pathways for ATP release in outflow and inflow cells. The inhibitors and, in parentheses, the release-pathway targets are presented along the abscissa. The numbers of wells studied are indicated for both outflow and inflow cells. $P2X_7$ receptors mediated ATP release only in TM cells, whereas vesicular ATP release was displayed only by ciliary epithelial (CE) cells.

This view has recently been supported by two new observations. First, gene expression of ectoATPases that metabolize ATP to adenosine [40] has been found [20] in both explant-derived human TM cells and the human TM5 cell line [41]. These ectoenzymes include: E-NPP1-3, members of the ecto-nucleotide pyrophosphatase/phosphodiesterase family converting ATP to AMP; E-NTPD2 and E-NTPD8 of the ecto-nucleoside triphosphate diphosphohydrolases converting both ATP to ADP and ADP to AMP; and ecto-5'-nucleotidase (CD73) converting AMP to adenosine. The identification of these six ectoATPases documents the plausibility that ATP release can modulate adenosine delivery to the ARs. Second, addition of ATP to the solution bathing TM5 cells stimulated MMP secretion, which was strongly blocked by inhibiting metabolism of ATP to adenosine (Li, A. et al., manuscript submitted). Given this experimental support, we have focused on identifying the mechanisms and regulation of ATP release by ciliary epithelial inflow cells and TM outflow cells.

Mechanisms of ATP Release

Multiple pathways have been documented to subserve ATP release [42], including channels, transporters such as P-glycoprotein, vesicular release and cell lysis. Under physiologic conditions, ATP is expected to be largely in the form of $MgATP^{2-}$, with a minor fraction as ATP^{4-}, so that channels must have large-diameter conduits of at least ~12 Å [42]. Early evidence suggested that members of the ATP-binding cassette family such as the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) [43, 44] or P-glycoprotein [45, 46] might serve as conduits. Several additional candidates have been subsequently considered [42, 47], including the Volume-Sensitive Outwardly-Rectifying (VSOR) Cl^- channel, the maxi-Cl^- channel, and the connexin (Cx) and pannexin (Px) "hemichannels" and $P2X_7$ ATP ionoreceptors described below. P-glycoprotein is an unlikely candidate since antibodies blocking whole-cell currents do not affect ATP release in HTC-R and NIH3T3/MDR1 cells over-expressing the protein [42]. The swelling-activated anion channel (aka VSOR, VRAC and VSAC) has been excluded since ATP release and activation of this channel can also be dissociated [42]. In contrast, the maxi-Cl^- channel is a plausible pathway. Although the molecular structure of the maxi-Cl^- channel is unknown [48], its inner bore radius is ~13 Å, roughly twice that of ATP.

The term "hemichannels" is commonly applied to unpaired wide-bore channels that either form [connexins (Cxs), innexins] or were thought to form [pannexins (Pxs)], gap junctions in paired cells. Innexins form both gap junctions and hemichannels in protosomes, including nematodes, and connexins subserve the same dual function in deuterostomes, including vertebrates [49]. Connexin hemichannels have long been known [50]. More recently, the pannexins (Px1-3) have been identified [51]. The Pxs, Cxs and innexins share a common topology [52], but there is only a 20% homology between Pxs and innexins and no homology between Cxs and either Pxs or innexins. Although Px1, like Cxs, was initially thought to form gap junctions, the four (rather than six) cysteine sites in the extracellular loops and the rich glycosylation of pannexin proteins are currently thought to preclude Px-based gap-junctional formation [49, 52].

The role of Px1 as a conducting channel and conduit for ATP release has been documented by several laboratories [49, 53-56]. The physiologic significance of Px2 as a homomeric hemichannel is less certain. Bruzzone et al. [57] found Px1 activity but no homomeric Px2 activity following heterologous expression of rat Px1 in Xenopus oocytes. Co-expression of Px1 and Px2 produced reduced currents that gated more slowly in comparison to the homomeric Px1 currents, suggesting that Px1/Px2 heteromeric channels had been formed. In contrast, Ambrosi et al. [52] reported that heterologous expression solely with a longer rat Px2 construct did produce currents without concurrent overexpression of Px1. However, the Px2-associated currents could not be stimulated at physiologically-relevant intracellular potentials, so that the potential *in vivo* importance of these currents is unknown. The final member of the Px family, Px3, has not been associated with channel activity [57]. Recent data suggest that Px3 plays a role in chondrocyte differentiation [58] and bone development [59].

ATP release is also associated with stimulation of the ionotropic ATP receptor $P2X_7$. The cationic conduit of $P2X_7$ is opened within milliseconds of its activation, but goes on to become permeable to large cations within subsequent seconds during continued stimulation [60]. $P2X_7$ has been reported to trigger activation of both PX1-mediated and PX1-independent large-bore channels [61]. Whether the large-bore conduit associated with $P2X_7$ is intrinsic to the receptor [62] or reflects the associated PX1 hemichannel [63, 64] has been controversial [60].

Many widely-applied blockers exert significant cross-channel inhibition [47], but several relatively selective agents have proved informative in pharmacologically distinguishing the ATP-releasing pathways. Probenecid inhibits PX1, but not Cx hemichannels [65]. The (-)-threo-(11R/2R) diastereomer of mefloquine also blocks PX1 with much higher potency than Cx hemichannels [66]. In contrast, both 1 mM heptanol [55] and extracellular Ca^{2+} [53] inhibit Cx, but not PX1, hemichannels. We have used bafilomycin A1 (BAF) to inhibit vesicular ATP release (Fig. 2). BAF acts by interfering with vesicular uptake of ATP, and is widely applied to block exocytosis of ATP [19]. We have complemented the pharmacologic approach with knockdown [18] and overexpression [18, 19] strategies in identifying the principle ATP release pathways in HEK293T and TM cells.

ATP Release Pathways from Trabecular Meshwork Outflow Cells

Both human explant-derived native TM cells and the human TM5 cell line displayed gene expression of multiple ATP-releasing conduits. PX1, Cx26, Cx31, Cx43, the $P2X_7$ ionotropic ATP receptor, and CFTR were identified by the reverse-transcription polymerase chain reaction (RT-PCR). Responses observed to separate application of ~20 inhibitors to TM5 cells indicated that PX1 hemichannels, Cx hemichannels and $P2X_7$ receptors provided comparable contributions to ATP release, measured by the luciferin-luciferase reaction (Fig. 2). CFTR, swelling-activated anion channels and vesicular release played no significant role. Simultaneous block of PX1, Cx and $P2X_7$ pathways inhibited ATP release by more than 96 ± 1%, so that cell lysis had a negligible effect. Identical responses were found in explant-derived TM cells after exposure to a selected number of inhibitors [18].

Estimation of the relative importance of the PX1, Cx and $P2X_7$ pathways in releasing ATP was based on the pharmacologic responses (Fig. 2). The validity of this approach was tested by examining the pharmacologic profile after partial knockdown of PX1 hemichannels with a lentiviral shRNA strategy. As expected, PX1 knockdown reduced total ATP release and the efficacy of the PX1-blocker probenecid in further inhibiting release. In contrast, PX1 knockdown enhanced the percentage inhibition produced by blocking either Cx or $P2X_7$ pathways of the residual ATP release [18].

Blocking PX1 and Cx pathways concurrently produced an additive inhibition of swelling-activated ATP release [18]. However, concurrent block of $P2X_7$ together with either PX1 or Cx hemichannels produced a less-than-additive inhibition [20]. Thus, the contribution of $P2X_7$ to ATP release appears greater when ATP is being concurrently released through parallel pathways. This view is consistent with the expectation that activation of the $P2X_7$ ATP receptor will be greater in the presence of higher baseline ATP levels.

ATP Release Pathways from Ciliary Epithelial Inflow Cells

Primary cultures of bovine mixed NPE and PE (bCE) cells and transformed bovine NPE and PE cell lines were found by RT-PCR to express PX1-3, Cx43, Cx40 and $P2X_7$. Expression of message for PX1 was confirmed by northern blots. Expression of bovine PX1 in HEK293T cells enhanced swelling-activated ATP release, and that increase was inhibited by the PX1 blocker probenecid [19].

Like TM cells, swelling-activated native and transformed bovine ciliary epithelial cells released ATP through three major pathways (Fig. 2). Similarly to TM cells, ciliary epithelial cells also utilized PX1 and Cx hemichannels. However, in contrast to TM cells, $P2X_7$-mediated release was insignificant. Instead, approximately 20% of the release was vesicular (Fig. 2). Concurrent blockade of PX1, Cx and vesicular release pathways inhibited swelling-activated ATP release by >90% [19].

The foregoing observations suggest that applying blockers of vesicular release to the eye's anterior segment might lead to inhibition of inflow without affecting outflow resistance, thereby lowering IOP. We are pursuing this possibility, but two caveats must be kept in mind. First, because of the difficulty in obtaining very fresh post-mortem human ciliary epithelial cells, the inflow cells studied were bovine. Second, the studies described have been conducted with isolated cells. The regulatory volume responses, and perhaps other responses, of individual ciliary epithelial cells have proved to be highly region dependent, at least in rabbit ciliary epithelium [67].

Modulators of ATP Release

The regulation of ATP release is incompletely understood. At least six modulators of release have been implicated: cell volume, mechanical perturbation, redox state, intracellular Ca^{2+}, growth factors, and cytoskeletal remodeling. Hypotonic cell swelling [68] and mechanical perturbation [54, 69, 70] are well-documented triggers of ATP release. In addition to measuring swelling-activated ATP release, stretching TM5 cells by 30% enhanced ATP release by twofold [20]. Separately blocking PX1, Cxs, $P2X_7$ and vesicular release produced the same percentage inhibitions in stretch-activated ATP release as those observed in swelling-activated release. Combined inhibition of PX1, Cxs and $P2X_7$ almost completely abolished stretch-activated ATP release. Evidently, cell swelling and mechanical perturbation trigger identical signaling pathways for ATP release in TM5 cells.

The redox state of the cell also modulates ATP release insofar as the reducing agent dithiothreitol (DTT) partially inhibits PX1 channels, an inhibitory effect that is precluded by interaction of PX1 with the K^+ channel subunit Kvb3 [71]. In TM5 cells, DTT alone reduced swelling-activated ATP release by 26.4 ± 1.2%, but had no further effect when added to cells exposed to the PX1 blocker probenecid [18]

Whether or not intracellular Ca^{2+} activates PX1 has been controversial. Single-channel records of *Xenopus* oocytes heterologously expressing PX1 indicate that PX1 is activated within seconds of applying micromolar concentrations of cytosolic Ca^{2+} to inside-out patches [72]. In contrast, intracellular Ca^{2+} did not affect PX1 channels expressed in mammalian cells [55]. In TM5 cells, exposure to ionomycin did enhance ATP release but only after a prolonged lag time, 7-8 times longer than after hypotonic challenge. That release was abolished by blocking $P2X_7$ with the large organic cation KN-62 [73] applied to either PX1-knockdown or mock-knockdown TM5 cells, but not by the PX1 inhibitor probenecid [18]. Evidently, change in cytosolic Ca^{2+} activity over the physiologic range does not activate PX1 hemichannels in TM cells. The $P2X_7$-mediated increase triggered by ionomycin may reflect apoptotic magnification of ATP release initiated by ATP permeating membrane that was compromised by the high intracellular Ca^{2+} activity [18].

Growth factors have been reported to stimulate ATP release in other cells. Basic fibroblast growth factor (FGF-2) activates Cx hemichannels in Cx43-expressing C6 glioma cells [74]. Acidic fibroblast growth factor (FGF-1) initiates a series of events leading to stimulation of vesicular release and later of $P2X_7$ receptors, and Cx and PX hemichannels in rat spinal astrocytes [75].

Remodeling of the actin cytoskeleton has been found to modulate swelling-evoked ATP release from TM5 cells [20]. Cytochalasin D-triggered depolymerization enhances, whereas prolonged exposure to dexamethasone reduces, swelling-activated ATP release. The effects of cytoskeletal modification were partly mediated by modulation of the duration of cell swelling after applying hypotonicity. The modulation of ATP release may regulate MMP secretion by adjusting ectoenzymatic generation of adenosine, activating A_1ARs and thereby leading to MMP-mediated modification of outflow resistance.

In addition to these six modulators, we have very recently found that swelling-activated ATP release is also reduced by exposure to low concentrations of ouabain (≥10 nM) or to equivalent concentrations of the aglycone strophanthidin (Li et al., manuscript submitted). The effect on ATP release could be dissociated from the effects of the cardiotonic steroids on ion-exchange activity of the target, Na^+,K^+-activated ATPase, suggesting mediation through scaffolding and/or signaling functions of the enzyme [76, 77].

Conclusions

Adenosine receptors (ARs) of the inflow and outflow aqueous humor pathways exert opposing actions on IOP. Adenosine delivery to A_3ARs in the ciliary epithelium activates Cl^- channels, leading to increased IOP. In contrast, adenosine delivery to A_1ARs in the TM cells stimulates MMP secretion, reducing resistance to aqueous humor outflow and decreasing IOP. In each case, adenosine is delivered by ectoenzymatic conversion of ATP released from the cells. In both inflow and outflow cells, PX1 and Cx hemichannels play major roles in ATP release. However, vesicular release is a major ATP-release mechanism only in the ciliary epithelial inflow cells, whereas $P2X_7$ subserves ATP release only from human TM outflow cells (Fig. 2). This difference in ATP release mechanisms suggests that blocking vesicular release might selectively inhibit inflow without altering outflow resistance, thereby lowering IOP.

Although understanding of the regulation of ATP release is incomplete, release from TM cells is increased by cell swelling, cell stretch, and actin depolymerization. ATP release is inhibited by the reducing agent dithiothreitol. Fibroblast growth factors increase ATP release in other cells. Elevated intracellular Ca^{2+} has been reported to increase PX1 activity in some, but not other, cells. In TM5 cells, increasing cytosolic Ca^{2+} does not increase ATP release through PX1 hemichannels. Finally, cardiotonic steroids reduce swelling-activated ATP release, an effect likely mediated by scaffolding/signaling functions, rather than by the ion-exchange role, of Na^+,K^+-activated ATPase.

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