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Hyposmotic stress causes ATP release in a discrete zone within the outer cortex of rat lens

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Purpose: Purinergic signaling pathways activated by extracellular ATP have been implicated in the regulation of lens volume and transparency. In this study, we investigated the location of ATP release from whole rat lenses and the mechanism by which osmotic challenge alters such ATP release.

Methods: Three-week-old rat lenses were cultured for 1 h in isotonic artificial aqueous humor (AAH) with no extracellular Ca^{2+}, hypotonic AAH, or hypertonic AAH. The hypotonic AAH-treated lenses were also cultured in the absence or presence of connexin hemichannels and the pannexin channel blockers carbenoxolone, probenecid, and flufenamic acid. The ATP concentration in the AAH was determined using a Luciferin/luciferase bioluminescence assay. To visualize sites of ATP release induced by hemichannel and/or pannexin opening, the lenses were cultured in different AAH solutions, as described above, and incubated in the presence of Lucifer yellow (MW = 456 Da) and Texas red-dextran (MW = 10 kDa) for 1 h. Then the lenses were fixed, cryosectioned, and imaged using confocal microscopy to visualize areas of dye uptake from the extracellular space.

Results: The incubation of the rat lenses in the AAH that lacked Ca^{2+} induced a significant increase in the extracellular ATP concentration. This was associated with an increased uptake of Lucifer yellow but not of Texas red-dextran in a discrete region of the outer cortex of the lens. Hypotonic stress caused a similar increase in ATP release and an increase in the uptake of Lucifer yellow in the outer cortex, which was significantly reduced by probenecid but not by carbenoxolone or flufenamic acid.

Conclusions: Our data suggest that in response to hypotonic stress, the intact rat lens is capable of releasing ATP. This seems to be mediated via the opening of pannexin channels in a specific zone of the outer cortex of the lens. Our results support the growing evidence that the lens actively regulates its volume and therefore, its optical properties, via puerinergic signaling pathways.

The maintenance of lens transparency is critical for the correct focusing of light onto the retina. The mammalian lens is a multicellular organ that consists of a single layer of cuboidal epithelial cells which cover the anterior surface of the lens. At the equator of the lens, these epithelial cells continually divide and differentiate into elongated fiber cells that comprise the bulk of the lens [1]. Studies have shown that cell volume regulation in lens epithelial and fiber cells is critical for maintaining lens transparency [2]. Both lens epithelial cells and fiber cells abundantly express ion and water channels as well as ion transporters to actively regulate their volume [3-7]. These epithelial and fiber cells are functionally coupled by gap junction channels [8,9], so the volume of the whole lens is maintained by the synchronized activities of the ion channels and transporters localized in different regions of the lens [2]. When lens volume regulation is impaired due to chronic or excessive osmotic stress (e.g., caused by diabetes or hypotonic stress) or pharmacological manipulation of ion channel or transporter activity, fiber cell swelling or extracellular space swelling in the outer cortex occurs, which leads to scattering of light and loss of lens transparency [6,10-12].

Our group and others have provided evidence that purinergic signaling pathways are involved in the regulation of the lens volume. We have mapped the expression of purinergic receptors in rat lens and showed that the lens expresses a range of P2 ($P2Y_{1,2,4,7,6}$ and $P2X_{1–7}$) receptors [13,14] and that P2X receptor activation under volume regulatory stress may contribute to the localized fiber cell swelling observed in the outer cortex in diabetic cataract [15,16].

Previous studies have shown that while ATP can be released from ciliary epithelial cells [17], corneal endothelial cells [18], and retinal epithelial cells [19,20], the lens is also a source of ATP release. Cultured human lens epithelial cells release ATP in response to hypertonic stress [21], and porcine lenses release ATP in response to hypotonic stress [22]. While the mechanism of ATP release is thought to be linked to connexin hemichannels and pannexin channels, this has not yet been investigated. It is known that the lens

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abundantly expresses Cx43 [23], Cx46 [24,25], and Cx50 [26] and that connexin proteins also exist as connexon hemichannels outside the gap junction plaques [27,28]. Furthermore, it has been shown that removal of extracellular Ca^{2+} can induce activation of the Cx46 [28,29] and Cx50 [30,31] hemichannels in lens fiber cells. The pannexin isoforms Panx1 and Panx2 have been identified in mouse lens [32], and while the physiologic function of pannexins in the lens is currently unknown, it is known that pannexins form channels in *Xenopus* oocytes and can release ATP [33], and that Panx1 is involved in ATP release from mouse taste buds [34] and in human erythrocytes [35].

To date, studies on ATP release in the lens have largely focused on lens epithelial cells [21,36]. Therefore, in this study, we extended our investigation to examine ATP release from intact rat lenses by using the uptake of Lucifer yellow (LY) to visualize openings of connexin hemichannels and pannexin channels under different volume regulatory stress conditions and to test the involvement of connexins and pannexins in mediating ATP release. Our data suggested that cortical fiber cells respond to hypotonic stress by opening pannexin channels that release ATP, which can then activate purinergic signaling pathways to restore fiber cell and overall lens volume so as to maintain the optical properties of lenses.

METHODS

Animals and reagents: Nineteen- to 23-day-old male and female Wistar rats were killed by CO_2 asphyxiation followed by cervical dislocation using protocols approved by the University of Auckland Animal Ethics Committee and which adhered to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. The whole eyes were removed immediately after death, and the lenses were extracted by making incisions on the posterior sclera. Then the lenses were placed into artificial aqueous humor (AAH: 125 mM NaCl, 0.5 mM $MgCl_2$, 4.5 mM KCl, 10 mM $NaHCO_3$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM sucrose, 10 mM HEPES, pH 7.2–7.4, 300±5 mOsmol). Unless otherwise stated, all the chemicals were purchased from Sigma-Aldrich.

In vitro culturing of the lenses: The dissected lenses were immediately placed in a 24-well culture plate that contained 2 ml of pre-warmed AAH and 1% penicillin/streptomycin/neomycin per well, and incubated at 37 °C. After 1 h, the lenses that developed opacities were discarded. After another 1 h of pre-incubation, the lenses were carefully transferred to wells that contained 1 ml of either isotonic AAH, 40% hypotonic AAH, 40% hypertonic AAH, or 0 mM $[Ca^{2+}]_e$ AAH (Table 1). Then the lenses were incubated at 37 °C for 1 h, throughout which period up to eight samples of 20.5 µl of the media were collected from each well. At the end of each incubation, 100 µl samples of the media were also collected and stored at 4 °C for lactate dehydrogenase (LDH) determination as a measure of tissue integrity. To inhibit ATP release, varying concentrations of the following known connexin hemichannel/pannexin channel inhibitors were added to the AAH-based media during both the pre-incubation and subsequent 1 h incubation [37-39]: carbenoxolone (CBX, 10 µM, 20 µM, or 50 µM dissolved in AAH) [37,40]; flufenamic acid (FFA, 5 µM, 10 µM, or 20 µM dissolved in dimethyl sulfoxide [DMSO]) [37,41], or probenecid (0.1 mM, 1 mM, or 3.5 mM dissolved in DMSO) [38,39]. In the control experiments, the lenses were incubated in the same concentrations of the vehicles used to dissolve the different pharmacological reagents. To assess the viability of the cultured lenses, an LDH assay was employed, as previously established [42]. After the 1 h incubation, the LDH levels in 100 µl of the AAH samples were assessed following the manufacturer's instructions (Roche Applied Science, Roche Diagnostics Corp.). The lenses that exhibited opacities during the first hour of pre-incubation, or the lenses with LDH activity greater than the adopted threshold of 0.05 units/ml, were deemed damaged and excluded from subsequent analyses.

D-Luciferin/Luciferase ATP release assay: A D-luciferin/luciferase assay kit (Molecular Probes™, Invitrogen) was used in accordance with the manufacturer's protocols to determine the amount of ATP released from the lenses into the culture media. For each experiment, a fresh standard curve was constructed from a series of ATP concentrations (0 nM, 5 nM, 25 nM, 50 nM, 100 nM, 150 nM, and 200 nM), which were diluted in AAH. Luminescence reading parameters, such as sensitivity and reading duration, were optimized using ATP standard solutions. A volume of 90 µl of the standard reaction solution was mixed with either 10 µl of the sample or the ATP standard solution in an Optiplate white 96-well plate (PerkinElmer™, MA). The measurements were recorded using a BioTek® Synergy 2 Microplate reader (BioTek Instruments, Inc., VT). The ATP concentrations from the collected samples were determined based on the standard curve measurements. The Invitrogen™ standard reaction solution was prepared fresh on the day of the experiment, and care was taken to minimize the exposure of the reaction solutions to light and to ensure that the mixing was gentle to avoid denaturation of the firefly luciferase.

Dye uptake assay: To determine the location and mechanism of ATP release in the lens, 2 mM of Lucifer yellow (LY, 456 Da MW) and 2.5 µM of Texas red dextran (TRD, 10 kDa MW) were added to the different incubation media listed in

Table 1. Immediately after the incubation, the lenses were fixed in 0.75% paraformaldehyde in PBS (final pH 7.0–7.5) for 24 h at room temperature and then cryoprotected and cryosectioned following protocols previously established in our laboratory [43]. The cryosections were washed three times in PBS and mounted with an anti-fading reagent (Citifluor™ AF100, Citifluor Ltd.). All the images were taken using a Leica Confocal Laser Scanning Microscope (SP2, Leica Microsystems, Wetzlar) or an Olympus Fluoview FV1000 Confocal Laser Scanning Microscope System (Olympus, Japan). Sequential scanning was used to separately obtain signals from TRD and LY to avoid bleed through from the two channels. The images were processed using Adobe Photoshop 2020 (Adobe Inc., CA) or analyzed using ImageJ.

Statistical analysis: Statistical analysis was performed using the computer software SPSS Statistics (Version 27.0, SPSS Inc., IL). In all the analyses, a p value of less than 0.05 was deemed statistically significant. For the analysis of the measurement of extracellular ATP, the univariant mixed-model analysis of variance (ANOVA) was used to compare ATP concentrations in the culture medium after 1 h of incubation between different treatment groups across the entire experiment (n = 312). The p value between each treatment group compared to the isotonic control group was obtained from Turkey's post-hoc analysis following ANOVA. Graphs were plotted using Microsoft Excel or GraphPad Prism (Version 9.2, CA),

RESULTS

Removal of extracellular Ca^{2+} induces ATP release and dye uptake in the rat lenses: Since previous studies had shown that epithelial cells were able to release ATP using a mechanism that involved the activation of hemichannels [36], we wanted to see if a similar mechanism was also operating in lens fiber cells. To test the feasibility of making these measurements in whole lenses, we first investigated whether culturing lenses in the absence of extracellular Ca^{2+}, $[Ca^{2+}]_e$, a perturbation that is known to activate the fiber-specific Cx46 hemichannels expressed in oocytes [25] and in isolated fiber cells [28], would be able to induce ATP release in whole lenses. The lenses were incubated in isotonic AAH that contained either 2 mM or 0 mM Ca^{2+} for 1 h, after which the ATP levels in the media were measured every 10 min. We observed that in the lenses cultured in isotonic AAH that contained 2 mM $[Ca^{2+}]_e$, relatively constant baseline levels of ATP release were detected in the media throughout the 1 h period (Figure 1A). In contrast, in the absence of $[Ca^{2+}]_e$, the lenses exhibited a significant release of ATP into the bathing medium after as early as 20 min, which subsequently increased over the remaining period of incubation (Figure 1A). We observed that at the 60-min time point, the release of ATP was 30X greater in the 0 mM $[Ca^{2+}]_e$ AAH relative to the isotonic AAH that contained 2 mM Ca^{2+} (Figure 1B). This increase in ATP release induced by the removal of $[Ca^{2+}]_e$ was considered not the result of a general loss of membrane integrity, since the LDH measurements showed low levels of LDH activity in the media of the lenses incubated without $[Ca^{2+}]_e$ (data not shown).

To investigate from where in the lens the ATP detected in the media originated and whether this release was mediated by the opening of hemichannels, we monitored the penetration of two fluorescent dyes, LY (MW = 456 Da) and TRD (MW = 10,000 Da), into the lens. Under normal conditions, both dyes should be confined to the extracellular space of the lens. However, the molecular weight of LY at 456 Da is below the molecular cutoff of 1,000 Da for permeation through channels formed from either connexin hemi-channels or pannexin channels [44,45], and hence, the intracellular accumulation of a small molecule dye such as LY can be used as an indicator of channel activation. In contrast, with a molecular weight of 10,000 Da, TRD is above this permeation cutoff, and therefore, its intracellular accumulation is indicative of nonspecific cell membrane rupture. The lenses were cultured for 1 h in either isotonic AAH that contained 2 mM Ca^{2+} or zero $[Ca^{2+}]_e$

Table 1. Artificial aqueous humor.

Culture medium	Constituent (mM)	pH Osmolarity (mOsmol/kg)
Isotonic AAH	125 NaCl, 0.5 MgCl2, 4.5 KCl, 10 $NaHCO_3$, 2 $CaCl_2$, 5 glucose,, 20 sucrose, 10 HEPES	7.2~7.4 300±5 Osmol/kg
Hypotonic AAH	75 NaCl, 0.5 MgCl2, 4.5 KCl, 10 $NaHCO_3$, 2 $CaCl_2$, 5 glucose, 20 sucrose, 10 HEPES,	7.2–7.4, 180±5 mOsmol
Hypertonic AAH	200 NaCl, 0.5 $MgCl_2$, 4.5 KCl, 10 $NaHCO_3$, 2 $CaCl_2$, 5 glucose, 20 sucrose, 10 HEPES,	7.2–7.4, 425±5 mOsmol
0mM $[Ca^{2+}]_e$ AAH	127 NaCl, 0.5 $MgCl_2$, 4.5 KCl, 10 $NaHCO_3$, 5 glucose, 20 sucrose, 10 HEPES, 10 EDTA,	7.2~7.4, 300±5 Osmol/kg

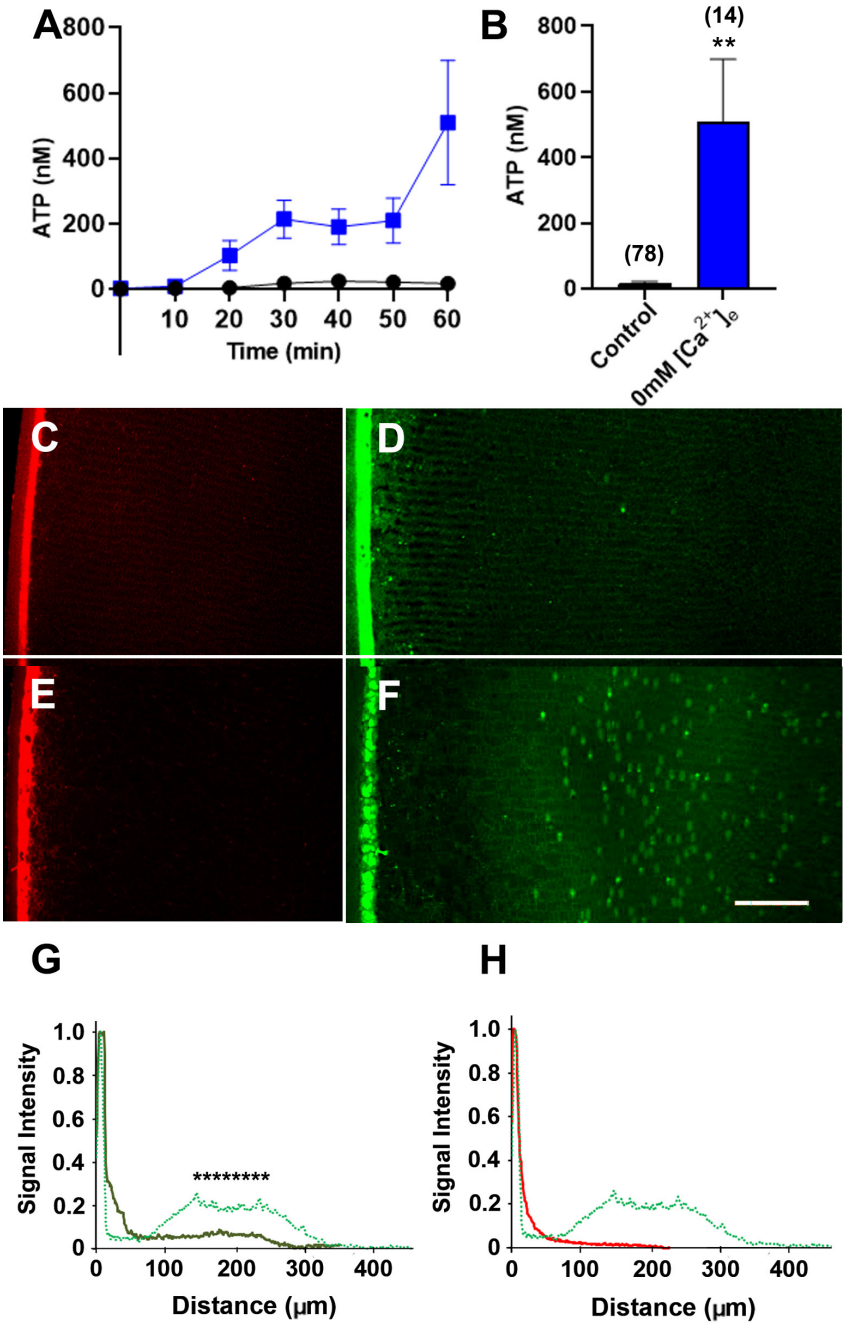

Figure 1. Removal of extracellular calcium induced Lucifer yellow (LY) uptake in lens fiber cells, which was correlated with ATP release. **A**, **B**: Time course of ATP release into the media measured from rat lenses incubated in either AAH (black) or 0 mM $[Ca^{2+}]_e$ AAH (blue). The error bars represent the standard error of the mean, and "()" represents the number of lenses in each experimental group. **p <0.01. **C**-**F**: Images of Texas red-dextran (TRD; C, E) and LY (**D**, **F**) penetration in equatorial sections from lenses incubated for 1 h in either the control AAH (C, D) or the 0 mM $[Ca^{2+}]_e$ AAH (**E**, **F**). **G**: Normalized LY signal intensity plotted against distance into the lens taken from the lenses incubated in AAH (*green line*) or 0 mM $[Ca^{2+}]_e$ AAH (dashed green line) showing an area (***) of LY uptake induced by 0 mM $[Ca^{2+}]_e$. (H) Normalized LY (dashed green line) and TRD (red line) signal intensities plotted against distance into the lens taken from the lenses incubated in 0 mM $[Ca^{2+}]_e$ AAH. Scale bar = 50 µm.

as well as LY and TRD, fixed, cryosectioned, and imaged via confocal microscopy to map the penetration of the two dyes into the outer cortex (Figure 1C–F). In the lenses that were incubated in the presence of 2 mM $[Ca^{2+}]_e$, both TRD (Figure 1C) and LY (Figure 1D) stained the porous capsule and labeled several epithelial cells that lay directly underneath the capsule; but in the deeper lenses, both dyes were restricted to the extracellular space between the rows of fiber cells. This penetration of LY and TRD into the rat lenses via the extracellular space is consistent with the results of previous studies in which both dyes freely diffused into the outer cortex of the lens before hitting a diffusion barrier located about 400 µm from the lens surface [46]. In the absence of extracellular Ca^{2+}, although LY and TRD staining was again detected in the capsule and epithelium at an intensity that was relatively equal to that in the presence of Ca^{2+}, there was a notable increase in the LY (Figure 1F) intensity but not in the TRD (Figure 1E) intensity in the fiber cells in the deeper regions of the outer cortex of the lens. To quantify this change in the dye intensity, plots of the signal intensity of the dye uptake with distance into the lens were extracted. Their analysis showed that the removal of extracellular Ca^{2+} caused an increased uptake of the low-molecular-weight dye LY in a discrete zone approximately 150–250 µm into the lens cortex (Figure 1G) but had no effect on the higher-molecular-weight TRD (Figure 1H). Since this zone of dye uptake occurred before the development of the extracellular diffusion barrier (~400 µm from the lens surface), the lack of TRD uptake was not related to a failure of TRD to reach this uptake zone. Taken together, these results suggest that the removal of extracellular Ca^{2+} activated the hemichannels in differentiating fiber cells in a discrete region of the lens, which is associated with a significant release of ATP into the media bathing of the lens. Having used a nonphysiologic stimulus to induce ATP release and validate our ability to visualize the location of that release in whole lenses, we next utilized the same approach to investigate the effects of osmotic stress on ATP release.

Hypotonic stress induces ATP release from intact rat lenses: The lenses were organ-cultured in either isotonic, hypotonic, or hypertonic AAH for 1 h with samples of media collected for ATP measurements at 10-min intervals over the 1 h period (Figure 2). While the osmotic challenge had no marked effects on the membrane integrity, as indicated by the absence of any significant changes in the LDH activity (*data not shown*), it did have significant effects on ATP release. Under isotonic conditions, ATP levels in the extracellular media showed a biphasic increase over time, which suggests that there is a basal level of ATP release under normal conditions (Figure 2A). Interestingly, it appeared that this basal ATP release was reduced in the lenses exposed to the hypertonic solutions (Figure 2A, B), but this decrease was deemed insignificant. At the 60-min time point, the ATP release significantly increased by 4.3X under the hypotonic conditions (70.55±8.47 nM, n = 47, p = 0.029) relative to the isotonic conditions (16.55±5.43 nM, n = 78; Figure 2A, B).

To visualize where in the lens this hypotonic-induced release of ATP was occurring, the lenses were organ-cultured in isotonic and hypotonic AAH and LY and TRD, fixed, sectioned, and imaged via confocal microscopy. To better appreciate where the dye uptake was occurring in the lens, the lenses were sectioned in an axial orientation to facilitate comparison of dye uptakes at the anterior pole and the equatorial regions of the lens. Under isotonic or hypotonic conditions, TRD was mainly restricted to the extracellular space between the epithelial and fiber cells at both the equator (Figure 2C and D) and the anterior pole (Figure 2C' and D'). The LY uptake was observed in the epithelial cells at the equator (Figure 2E) and the anterior pole (Figure 2E') under isotonic conditions but remained confined to the extracellular space between the fiber cells. However, while exposure to the hypotonic conditions had no noticeable effect on the LY uptake in the epithelium, it increased the uptake of LY in a discrete zone within the lens cortex (Figure 2F) that extended up toward the anterior pole (Figure 2F'). To quantify this change in dye intensity, plots of the signal intensity of the dye uptake with distance into the lens were extracted. Their analysis showed that exposure to hypotonic stress caused increased uptake of the low-molecular-weight dye LY in a discrete zone located approximately 150–300 µm into the lens cortex, with the LY signal starting to drop beyond 300 µm into the lens (Figure 2G), but had no effect on the higher-molecular-weight TRD (Figure 2H). Interestingly, the zone of the LY uptake induced by the hypotonic challenge, a known activator of hemichannels, was essentially similar to that seen in the lenses exposed to zero $[Ca^{2+}]_e$ (Figure 1G) [28-31]. Taken together, these results suggest that the fiber swelling induced by exposure of the lenses to hypotonic stress caused increased release of ATP mediated by the activation of hemichannels in differentiating fiber cells located in a discrete zone of the outer cortex of the lens. However, the identity of the ATP release channel remains to be determined.

Pharmacological inhibition of ATP release from the lens: To address the question of the molecular identity of the ATP release channel in the lens, a pharmacological approach that used CBX, FFA, and probenecid was adopted. CBX and FFA are known inhibitors of connexin hemichannels and, to a lesser extent, pannexin channels, and have been used to block connexin-hemichannel-mediated ATP release, whereas probenecid is a hemichannel blocker that has been shown to

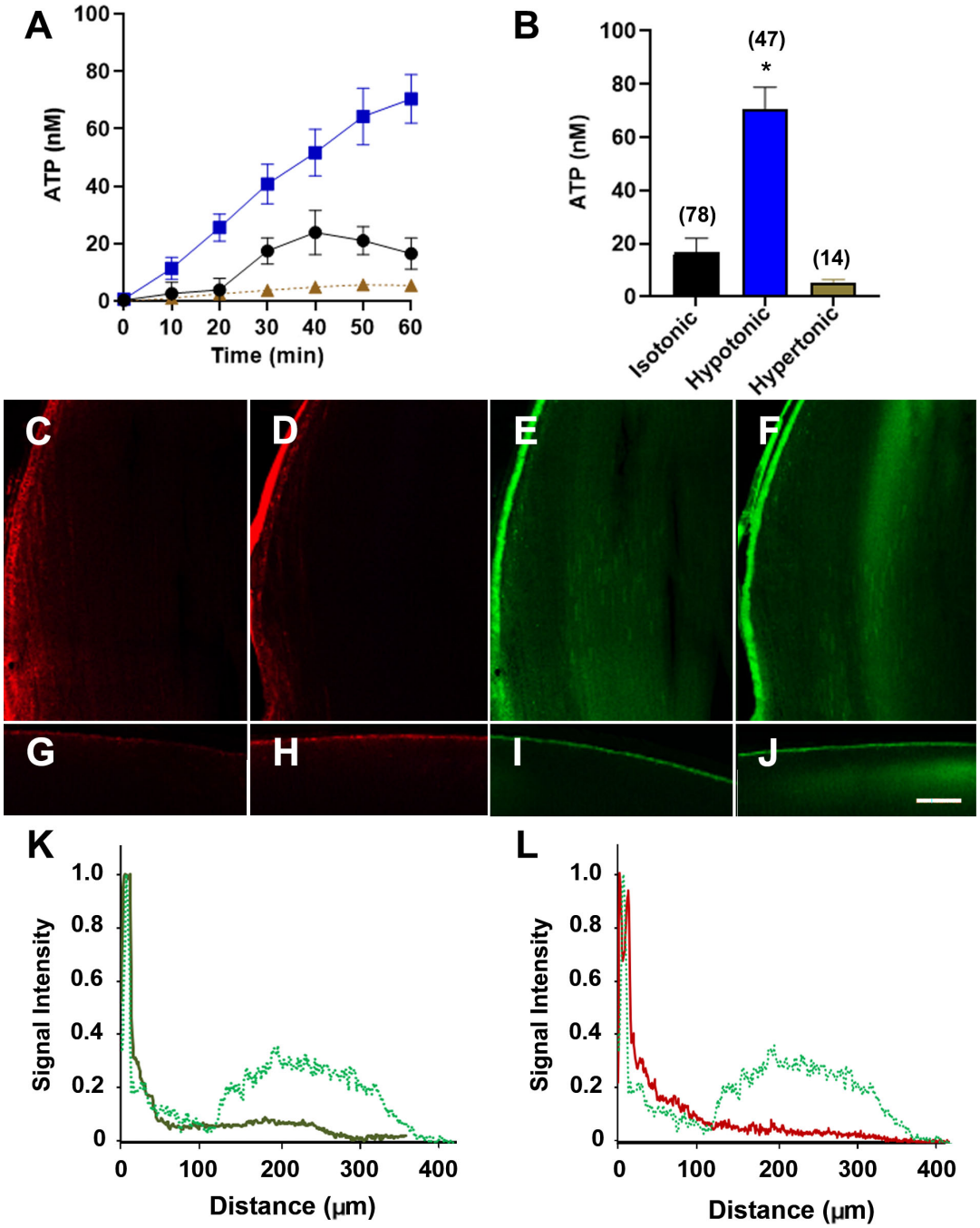

Figure 2. Hypotonicity induces ATP release from rat lenses in the lens cortex. **A**-**B**: Time course of ATP release into the media measured from the rat lenses incubated in either isotonic (300±5 mOsm black), hypertonic (420±5 mOsm brown), or hypotonic (175±5 mOsm blue) AAH for 1 h. The error bars represent the standard error of the mean, and "()" represents the number of lenses in each experimental group. *p < 0.01. **C**-**J**: Images of Texas red-dextran (TRD; **C**, **D**, **G**, **H**) and Lucifer yellow (LY; **E**, **F**, **I**, **J**) penetration in axial sections from the lenses incubated for 1 h in either isotonic (**C**, **E**, **G**, **I**) or hypotonic (**D**, **F**, **H**, **J**) AAH showing dye penetration orientation at the equator (C-F, top panels) and the anterior pole (**G**-**J**, bottom panels). (**K**) Normalized LY signal intensity plotted against distance into the lens taken from the lenses incubated in isotonic (green line) or hypotonic (dashed green line) AAH showing an area (***) of LY uptake induced by the hypotonic challenge. (**L**) Normalized LY (dashed green line) and TRD (red line) signal intensities plotted against distance into the lens taken from the lenses incubated in hypotonic AAH. Scale bar = 50 µm.

have a greater potency for pannexin channels than connexin hemichannels [38,40,47]. The rat lenses were cultured in hypotonic AAH in the absence (vehicle control) or presence of inhibitors for 1 h, with samples collected every 10 min over the 1 h period for ATP analysis.

The CBX used over a range of concentrations (10, 20, and 50 µM) appeared to have been ineffective in altering the time course of the ATP release induced by the hypotonic challenge (Figure 3A) and, in fact, appeared to have increased the release of ATP. However, analysis of the ATP levels at 60 min revealed that there was no statistical difference in the ATP release between the hypotonic conditions (70.55±8.47 nM, n = 47) and the hypotonic conditions plus CBX at all the concentrations tested: 10 µM (117.11±42.85 nM, n = 14), 20 µM (113.54±27.96 nM, n = 18), and 50 µM (118.54±49.76 nM, n = 9; Figure 3B). Similarly, the lenses cultured in hypotonic AAH + FFA at 5 µM, 10 µM, and 20 µM concentrations exhibited sustained release of ATP into the culture medium over the 60-min incubation (Figure 3C). After 60 min, the ATP concentrations in the culture media of hypotonic AAH + FFA at 5 µM (80.74±13.60 nM, n = 18), 10 µM (78.56±18.53 nM, n = 27), and 20 µM (189.66±39.53 nM, n = 16) did not significantly differ from the hypotonic + DMSO control (181.90±32.29 nM, n = 14; Figure 3D).

In contrast, both the 0.1 mM and 1 mM probenecid appeared to have reduced the ATP release over 60 min in a concentration-dependent manner (Figure 3E). After 60 min, the ATP concentrations in the culture media of hypotonic AAH + probenecid at 0.1 mM (105.91±27.35 nM, n = 9) and 3.5 mM (81.36±10.59 nM, n = 4) did not significantly differ from those in the culture medium of the hypotonic + DMSO control (181.90±32.29 nM, n = 14). However, in the presence of 1 mM probenecid, there was an 80% reduction in the ATP release compared to the control (39.20±12.61 nM, n = 11, p = 0.013), which was statistically significant (Figure 3E). Based on the effect of 1 mM probenecid on ATP release, we decided to use it to determine whether it could reduce the uptake of LY induced by a hypotonic challenge (Figure 4). While probenecid had no effect on the penetration of TRD into the lenses exposed to the hypotonic challenge for 1 h (Figure 4A, C and E), the hypotonic-induced uptake of LY by differentiating fiber cells, which occurred in the discrete zone that started ~150 µm from the lens surface (Figure 4B), was abolished by the addition of 1 mM probenecid (Figure 4D and 4F). The ability of 1 mM probenecid to inhibit ATP release and dye uptake induced by hypotonic stress suggests that ATP release is probably mediated by the pannexin channels in the lens.

DISCUSSION

Previous work by our group had shown that P2X and P2Y classes of purinergic receptors are expressed in the rat lens cortex [13,14]. In this study, we investigated whether whole lenses are capable of ATP release, which is required to induce autocrine regulation of the purinergic pathways identified in the outer cortex of the lens. To achieve this, we used a Luciferin/Luciferase assay to monitor the ATP release in order to visualize where in the lens the ATP release was occurring. To first validate this system, we used our knowledge of the sensitivity of connexin hemichannel gating to a reduction in extracellular Ca^{2+} in order to induce ATP release through the nonphysiologic opening of the hemichannels in the whole lens. Under these conditions of zero extracellular Ca^{2+}, a large increase in ATP release was observed (Figure 1A,B), which was localized to a distinct region of the outer cortex of the lens (Figure 1C–H), as seen in the uptake of the lower-molecular-weight LY but not of the larger TRD dye. Having evaluated our experimental approach using low extracellular Ca^{2+}, we next investigated the effects of osmotic challenge on the magnitude and location of ATP release. We found that even under isotonic conditions, there was a basal level of ATP release that appeared to be associated mainly with the epithelium (Figure 2). The exposure of the lenses to a hypertonic challenge appeared to have reduced this basal release of ATP, but this trend was insignificant (Figure 2B). In contrast, the hypotonic challenge caused a large and sustained increase in ATP release that was localized to the same region of the lens as that induced by the exposure of the lenses to low extracellular Ca^{2+} (Figure 2F,G), a nonphysiologic procedure known to activate the Cx46 and Cx50 hemichannels. Further investigation revealed that the ATP release (Figure 3) and the dye uptake (Figure 4) induced by the hypotonic challenge could be significantly reduced with the addition of probenecid.

Probenecid is thought to have a more potent inhibitory effect on Pannexin 1 channels than the hemichannels composed of Cx46 or 32 [38]. In addition, Pannexin 1 channels are also strongly blocked by CBX but not by FFA. In contrast, cell-to-cell gap junction channels and connexon hemichannels are both strongly inhibited by CBX and FFA [38,41,48]. Based on this pharmacological profile, it appears that pannexin channels are more likely to have been responsible for the observed ATP release in response to the hypotonic challenge, but in the absence of more specific pharmacological reagents that discriminate between pannexin channels and connexin hemichannels, we cannot rule out additional contributions from the Cx46 and Cx50 channels. Regardless of this uncertainty around the molecular identity of the ATP release hemichannel, our findings showed that

the lens constitutively releases ATP and that this release can be increased through local swelling of fiber cells that activate a channel that can mediate the release of ATP to the extracellular space where it can activate purinergic signaling pathways that control cell volume.

Our previous identification and localization of the P2X and P2Y classes of purinergic receptors in the rat lens cortex [13,14] showed that P2X receptors can be differentially recruited to specific membrane domains of lens fiber cells by osmotic and hyperglycemic stress within a discrete zone of the outer cortex region [16]. Interestingly, this region of the lens cortex involved in the recruitment of P2X isoforms to the

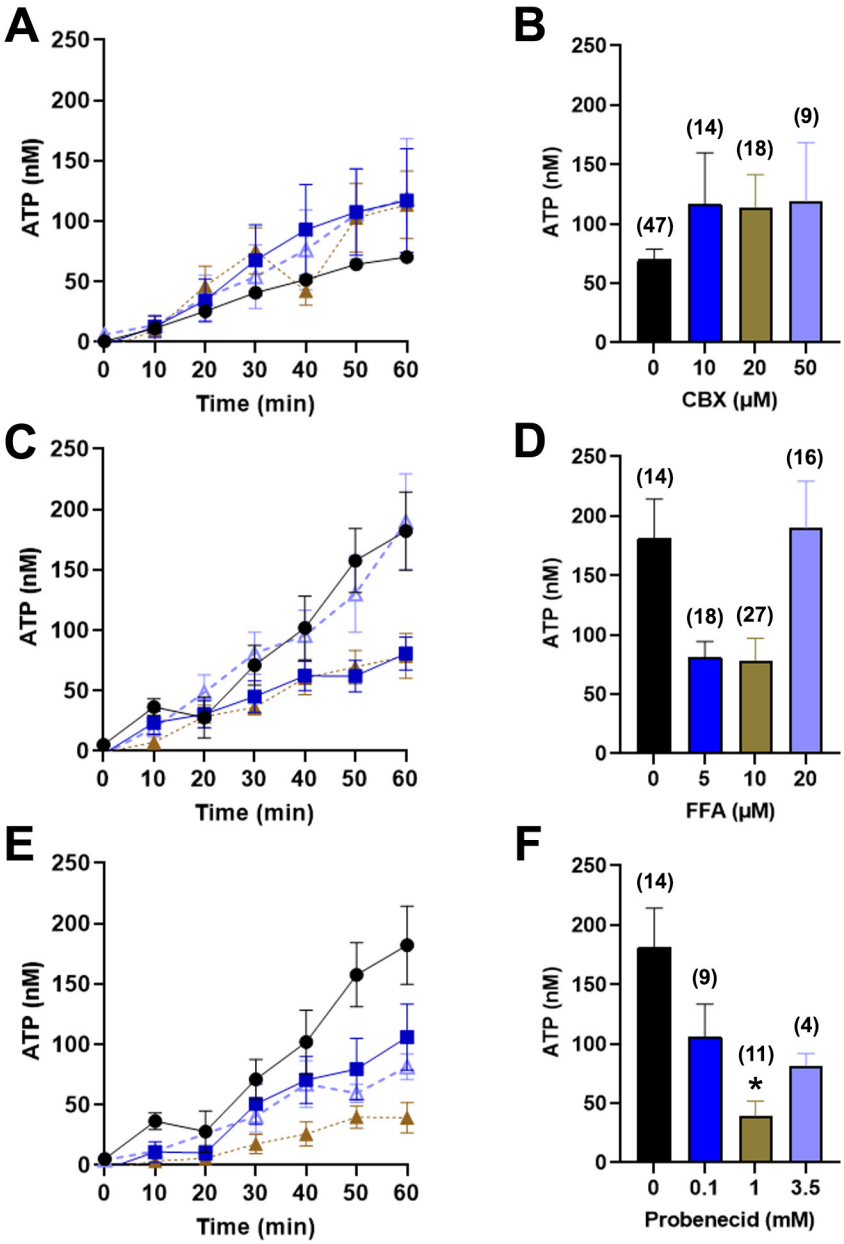

Figure 3. Effects of hemichannel inhibitors on ATP release induced by the hypotonic challenge. Time course of ATP release into the media measured from the rat lenses exposed to the hypotonic challenge in the absence or presence of the hemichannel inhibitors carbenoxalone (**A**, CBX), flufenamic Acid (**C**, FFA), or probenecid (**E**, Prob). Mean ATP release into the media from the rat lenses measured after 1 h of exposure to the hypotonic challenge in the absence or presence of CBX (**B**), FFA (**D**), or Prob (**F**). The error bars represent the standard error of the mean, and "()" represents the number of lenses in each experimental group. *p < 0.05.

membrane coincided with a zone of localized cell swelling in an in vivo diabetic rat model [10], and in rat lenses cultured in the presence of elevated extracellular glucose [49], which suggested the involvement of P2X purinergic receptors in the pathophysiology of diabetic lens cataract [50-54]. Work by Shahidullah et al. in the porcine lens showed that the P2Y class of purinergic receptors also plays a role in the volume regulatory response to hypotonic stress [22]. Porcine lenses also express the Transient Receptor Potential Cation Channel Subfamily V Member 4 (TRPV4) receptor, a mechano-sensitive receptor known to respond to membrane stretch [36,55], a view supported in the findings of Gao and others [56-58]. We likewise showed that mouse lens also express TRPV4 and TRPV1 isoforms of the TRPV family [59], and the receptor localization in the lens fiber cells dynamically respond to the zonular tension on the lens [59]. Shahidullah (2012) showed that TRPV4 is the mechanosensory receptor for hypotonic-induced cell swelling that can trigger an increase in intracellular Ca^{2+} and subsequently, stimulate Na^{+}/K^{+} ATPase activity to restore cell volume [22]. Intracellular Ca^{2+} can, in turn, influence gating of Panx 1 channels [60]. While the current study did not directly investigate P2X activation, we did show that hypotonic stress results in the opening of ATP release channels in the same region of the outer cortex where tissue liquefaction is observed in diabetic cataracts, providing further support for the involvement of purinergic signaling in the regulation of fiber cell volume. We speculate that hypotonic-stress-induced ATP release in the lens may be

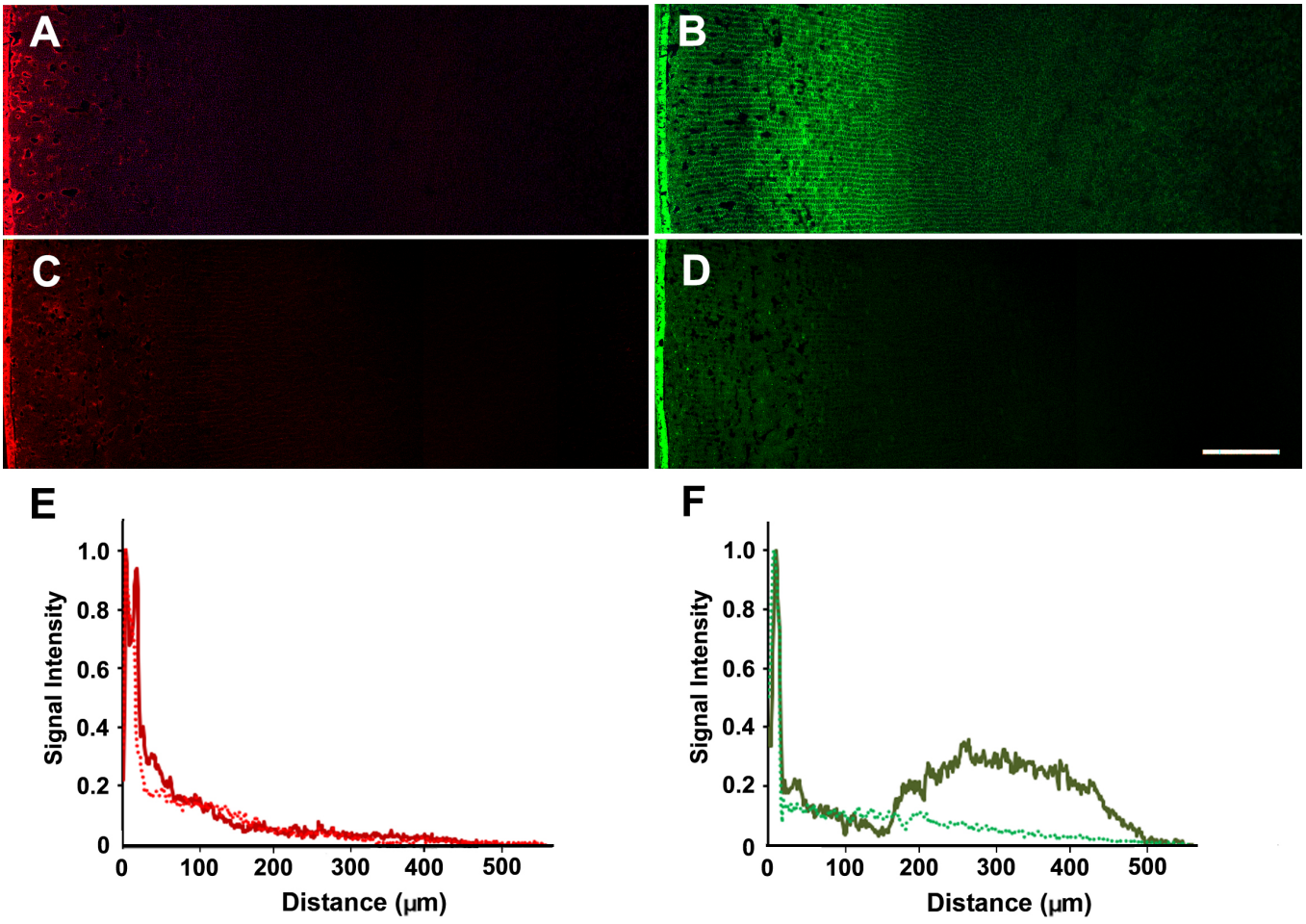

Figure 4. Effect of probenecid on Lucifer yellow (LY) uptake in lens exposed to the hypotonic challenge. Images of Texas red-dextran (TRD; **A, C**) and LY (**B, D**) penetration in equatorial sections from the lenses incubated for 1 h in either hypotonic AAH in the absence (**A, B**) or presence (**C, D**) of 1 mM probenecid. **(E)** Normalized TRD signal intensity plotted against distance into the lens taken from the lenses incubated in hypotonic AAH in the absence (red line) or presence (dashed red line) of 1 mM probenecid isotonic. **(F)** Normalized LY signal intensities plotted against distance into the lens taken from the lenses incubated in hypotonic AAH in the absence (green line) or presence (dashed green line) of 1 mM probenecid isotonic. Scale bar = 100 µm.

the net result of the activation of mechanosensitive TRPV4 channels and the subsequent Ca^{2+}-dependent opening of the Panx 1 channels that we know to exist in the mammalian lens. How components of this signaling pathway may work under physiology and pathological cell swelling, such as those induced by hyperglycemic conditions, will be of interest in the future.

In conclusion, we provided evidence of ATP release from intact rat lenses in response to hypotonic stress. Our results suggest that ATP release is mediated by pannexin channels and occurs in a distinct zone of the outer cortex. Investigations on how the swelling-induced opening of ATP release channels and the subsequent activation of ATP signaling pathways contribute to the pathology of diabetic cataract will be the focus of our future studies.

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