The Ciliary Muscle and Zonules of Zinn Modulate Lens Intracellular Hydrostatic Pressure Through Transient Receptor Potential Vanilloid Channels

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The zonules of Zinn are a circumferential system of fibrous strands connecting the lens to the ciliary body. The tension exerted by the zonules on the lens is modulated by the contraction or relaxation of the ciliary muscle. These forces produce accommodation in primate lenses, but could have additional effects in both accommodating and nonaccommodating species when sensed by the lens. The structure and composition of the mouse zonule are highly similar to that in humans, suggesting that forces transmitted by the ciliary muscle could serve novel signaling functions in the nonaccommodating mouse lens.2 The mouse eye also has a clearly defined ciliary muscle that expresses smooth muscle actin, caldesmon, and calponin, although it is smaller and less developed than the primate ciliary muscle, consistent with the reported lack of accommodation in mice.5,6

The transient receptor potential vanilloid (TRPV) family of channels are activated by a variety of stimuli, including stress induced by changes in osmolarity and/or pressure.2,7 At least two channels, TRPV1 and TRPV4, are expressed in the lens and their functional activity has been linked to ion homeostasis, water content, refractive index, and transparency.9–13 In many tissues, TRPV1 plays an opposing role to TRPV4 in osmoregulation,14,15 and this appears to be the case in the lens.12 TRPV4 channel activity in the lens epithelium caused increased Na+/K+-ATPase activity through a Src family kinase-dependent mechanism.3,16 In contrast, TRPV1 channels rapidly increase Na+/K+-ATPase activity through a mechanism dependent on phosphoinositide 3-kinase (PI3K) p110α knockout mice and immunostaining of phosphorylated protein kinase B (Akt), to determine how changes in ciliary muscle tension resulted in altered hydrostatic pressure.

RESULTS. Ciliary muscle relaxation increased the distance between the ciliary body and the lens and caused a decrease in intracellular hydrostatic pressure that was dependent on intact zonules and could be blocked by inhibition of TRPV4. Ciliary contraction moved the ciliary body toward the lens and caused an increase in intracellular hydrostatic pressure and Akt phosphorylation that required intact zonules and was blocked by either inhibition of TRPV1 or genetic deletion of the p110α catalytic subunit of PI3K.

CONCLUSIONS. These results show that the hydrostatic pressure gradient within the lens was influenced by the tension exerted on the lens by the ciliary muscle through the zonules of Zinn. Modulation of the gradient of intracellular hydrostatic pressure in the lens could alter the water content, and the gradient of refractive index.

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PURPOSE. Lenses have an intracellular hydrostatic pressure gradient to drive fluid from central fiber cells to surface epithelial cells. Pressure is regulated by a feedback control system that relies on transient receptor potential vanilloid (TRPV)1 and TRPV4 channels. The ciliary muscle transmits tension to the lens through the zonules of Zinn. Here, we have examined if ciliary muscle tension influenced the lens intracellular hydrostatic pressure gradient.

METHODS. We measured the ciliary body position and intracellular hydrostatic pressures in mouse lenses while pharmacologically causing relaxation or contraction of the ciliary muscle. We also used inhibitors of TRPV1 and TRPV4, in addition to phosphoinositide 3-kinase (PI3K) p110α knockout mice and immunostaining of phosphorylated protein kinase B (Akt), to determine how changes in ciliary muscle tension resulted in altered hydrostatic pressure.
Here, we have examined whether forces generated outside of the lens by the ciliary muscle could be transmitted to the lens through the zonules and affect hydrosstatic pressure. We found that the lens responded to these external forces with significant changes in the intracellular hydrosstatic pressure profile. These results show that the hydrosstatic pressure in the lens is regulated by the tension exerted by the ciliary muscle through the zonules, which could have a profound impact on the absolute water content and the gradient of refractive index within the lens.

**Materials and Methods**

**Imaging of the Ciliary Body and Lens**

Animal use conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Stony Brook University Institutional Animal Care and Use Committee. To preserve the native three-dimensional structure of the ciliary body, zonules, and lens, 2-month-old mouse eyes were fixed with 4% paraformaldehyde while maintaining intraocular pressure using the method described by Bassnett. After fixation, the posterior sclera and retina were removed and the ciliary body and lens were photographed using a SZX16 dissecting microscope, and the distance between them was measured using cellSens software (Olympus Corporation, Waltham, MA, USA).

**Lens Dissection**

Two-month-old wild-type, PTEN knockout, or PI3K p110α knockout mice on a C57BL/6J genetic background were euthanized and their eyes were enucleated and transferred to a petri dish containing normal Tyrode’s solution composed of (in mM) NaCl 137.7, NaOH 2.3, KCl 5.4, CaCl2 2, MgCl2 1, HEPES 5, and glucose 10, pH 7.4. To obtain lenses suspended by their zonules, the cornea, iris, and optic nerve were removed, and the sclera was cut into four flaps from the posterior surface. The lens was transferred and pinned by its sclera to the bottom of a Sylgard-coated chamber. To obtain lenses without attached zonules, four sclera insertions were made as above; the eye was then inverted inside out and a custom-made glass loop was used to carefully remove the lens from other ocular tissues and transfer it to the recording chamber. Lenses-containing chambers were mounted on a microscope stage and perfused with Tyrode’s solution.

**Measurement of Lens Intracellular Hydrostatic Pressure**

Intracellular hydrostatic pressures were measured at room temperature as previously described. Briefly, a voltage microelectrode having a resistance of 1.5 to 2.0 MΩ was filled with 3M KCl and positioned at a 45° angle between the posterior pole and equator of the lens. Tip resistance measurements were made in the normal Tyrode’s bathing solution by passing current pulses and recording the voltages responses. The microelectrode tip was advanced until an initial penetration at a position 20 to 30 μm from the lens surface was obtained. This resulted in an increased microelectrode tip resistance as fiber cell cytoplasm was pushed into the tip by the positive intracellular hydrostatic pressure. Pressure within the microelectrode was adjusted using a manometer connected to the side port of the holder until the cytoplasm was pushed out of the tip, and the resistance returned to its original value. The pressure required to return tip resistance to its initial value (in mm Hg) was recorded as the lens intracellular hydrostatic pressure at that specific location. Pressures were monitored for 30 minutes to establish a baseline. Following drug administration, pressure at that same location was monitored and recorded for an additional 100 minutes. In some experiments, intracellular pressures were recorded along a track at a 45° angle between the posterior pole and equator from the lens surface toward the center. Pressure was measured at four or five different depths in each lens, while recording the position of the tip of the microelectrode at each location. Data were pooled from several lenses and fit to a previously described structurally based model.

**Immunofluorescent Staining**

Lenses from eyes with intact zonules were dissected and transferred to Tyrode’s solution. The lens capsule was peeled away from the fiber cell mass using fine forceps, fixed in 1% paraformaldehyde in PBS for 1 hour, and blocked with 5% BSA dissolved in PBS with 0.02% NaN₃ and 0.1% Triton X-100 added. Capsules were immunostained with a rabbit monoclonal antibody against scrine 473 phospho-Akt (Cell Signaling Technology, Danvers, MA, USA) followed by a Cy3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Stained capsules were photographed on an Axiovert 200M microscope (Zeiss, Thornwood, NY, USA).

**Pharmacological Agents**

Tropicamide (0.1% wt/vol), or pilocarpine (0.2% wt/vol; both from Sigma-Aldrich Corp., St. Louis, MO, USA) was used to relax or contract, respectively, the ciliary muscle. HC067047 (10 μM; Tocris Bioscience, Bristol, UK) was used to inhibit TRPV4 channels. A889425 (10 μM; Alomone Labs, Jerusalem, Israel) was used to inhibit TRPV1 channels.

**Statistical Analysis**

Student’s t-test or 1-way analysis of variance (ANOVA) was used for 2- or 3-way comparisons of primary data, respectively (Originlab, Northampton, MA, USA).

**Results**

**Modulation of the Ciliary Muscle Altered the Distance Between the Ciliary Body and the Lens**

The muscarinic agonist pilocarpine, or the muscarinic antagonist tropicamide, was used to contract and relax the ciliary muscle, respectively, followed by fixation and measurement of the distance between the ciliary body and lens. The pupil diameter in control eyes (Fig. 1A) was visibly reduced following treatment with pilocarpine (Fig. 1B) and increased after tropicamide application (Fig. 1C), showing that the drugs resulted in smooth muscle contraction and relaxation, respectively. Removal of the posterior sclera, retina, and vitreous revealed a uniformly distributed circumlental space between the processes of the ciliary body and lens (Fig. 1D, asterisk) that contains the zonules of Zinn. In untreated control mouse eyes, the ciliary processes were located 149 ± 7 μm (Fig. 1E, arrow, mean ± SD, n = 10) from the lens, in good agreement with previously published values. In eyes pretreated for 30 minutes with 0.2% pilocarpine to contract the ciliary muscle, the ciliary processes appeared elongated, and the circumlental space was reduced to 124 ± 14 μm (Fig. 1E, n = 8). In eyes pretreated for 30 minutes with 0.1% tropicamide to relax the ciliary muscle, the ciliary processes appeared contracted, and the circumlental space was in-
The mean differences in circumlental space (H distance traveled by the microelectrode during impalement (to the flexibility of the lens capsule and variability of the intracellular pressure varied between 20 and 40 mm Hg due to the pupil dilation confirmed drug action. Removal of the posterior sclera, retina, and vitreous revealed the circumlental space between the ciliary processes and the lens (D, asterisk). Measurements taken on higher-power images showed that in control eyes (E), the distance between ciliary processes and the lens was 149 ± 7 μm (mean ± SD, arrow). In eyes treated with ciliary muscles contracted by pilocarpine (F), the circumlental space was reduced to 124 ± 14 μm. In eyes with ciliary muscles relaxed by tropicamide (G), the circumlental space was increased to 174 ± 7 μm. The mean differences in circumlental space (H) were statistically significant (P < 0.05, 1-way ANOVA, n = 6–10).

**Relaxation of the Ciliary Muscle Decreased Lens Hydrostatic Pressure**

Tropicamide was used to dilate the ciliary muscle and produce an outward (pulling) tension on the lens equator, while simultaneously recording hydrostatic pressure within a lens fiber cell near the lens surface. Initial values of surface intracellular pressure varied between 20 and 40 mm Hg due to the flexibility of the lens capsule and variability of the distance traveled by the microelectrode during impalement (n = 4). These initial values remained stable for 30 minutes until the application of 0.1% tropicamide to the bath solution, after which they were reduced by 20 to 25 mm Hg within 30 to 40 minutes (Fig. 2A). To better compare the data, pressures from individual lenses were normalized to their initial values (Fig. 2B) and plotted as the mean ± SD change in pressure during drug administration (Fig. 2C). On average, the relaxation of the ciliary muscle by tropicamide reduced the lens hydrostatic pressure near the surface by 24 ± 3 mm Hg (P < 0.05, Student’s t-test).

Intact zonules of Zinn were required for tropicamide to reduce the lens hydrostatic pressure. Lenses dissected free of the ciliary muscle and zonules had initial values of intracellular pressure of 24 ± 0.6 mm Hg after 100 minutes (Fig. 2C, white squares, P > 0.05), much less than the superimposed 24 ± 3 mm Hg reduction observed in lenses attached to the ciliary body (Fig. 2C, gray squares). Reduction of lens hydrostatic pressure by tropicamide also required the activity of the TRPV4 channel. Previous work has shown that TRPV4 responds to pressure changes as part of a feedback control system that maintains lens hydrostatic pressure at the surface at 0 mm Hg.12 When lenses with intact zonules were preincubated for 30 minutes in the TRPV4 inhibitor HC-067047,9 they had stable initial values of intracellular pressure of 24 ± 2 mm Hg. The addition of 0.1% tropicamide to the bath solution had no effect on pressure (Fig. 3C). On average, application of tropicamide to the TRPV4-inhibited lenses lowered the hydrostatic pressure near the surface by 1.9 mm Hg after 100 minutes (Fig. 3C). On average, application of tropicamide to the TRPV4-inhibited lenses lowered the hydrostatic pressure near the surface by 1.5 ± 1.9 mm Hg after 100 minutes (Fig. 3D, white triangles, P > 0.05), significantly less than the superimposed pressure change in lenses without TRPV4 inhibition (Fig. 3D, gray squares).

**Contraction of the Ciliary Muscle Increased Lens Hydrostatic Pressure**

Pilocarpine was used to contract the ciliary muscle, which moves the ciliary body forward and reduces the tension applied to the lens equator, while simultaneously recording hydrostatic pressure near the surface. The initial values of intracellular pressure at the lens surface varied between 18 and 30 mm Hg and remained stable for 30 minutes until the application of 0.2% pilocarpine to the bath solution, after which they increased by 13 to 18 mm Hg within 40 minutes (Fig. 4A). Contraction of the ciliary muscle by pilocarpine
increased the lens hydrostatic pressure near the surface by an average of 16 ± 3 mm Hg (Fig. 4B, P < 0.05).

Similar to the effect of tropicamide on reducing pressure, intact zonules were required for pilocarpine to increase the hydrostatic pressure. Lenses without attached zonules had initial surface intracellular pressure values of 20 ± 3 mm Hg that were unaffected by the addition of 0.2% pilocarpine to the bath (Fig. 5A). Application of pilocarpine to free lenses increased the hydrostatic pressure near the surface by a mean value of 1.3 ± 2.2 mm Hg after 100 minutes (Fig. 5B, white circles, P > 0.05), 92% less than the superimposed 16 ± 3 mm Hg increase in lenses with intact zonules (Fig. 5B, gray circles).

The increased lens hydrostatic pressure caused by application of pilocarpine required the activity of the TRPV1 channel. Previous work had shown that TRPV1 responds to lens hydrostatic pressure changes through stimulation of the Na+/K+/2Cl− cotransporter10 and an inhibitory effect on the Na+/K+ ATPase.12 Lenses with intact zonules preincubated in the TRPV1 inhibitor A-88942528 for 30 minutes had stable pressure values of 21 ± 2 mm Hg. The addition of 0.2% pilocarpine to the bath solution elicited no change in hydrostatic pressure (Fig. 5C). Application of pilocarpine to the TRPV1-inhibited lenses raised the hydrostatic pressure near the surface by 0.2 ± 1.2 mm Hg after 100 minutes (Fig. 5D, inverted triangles, P > 0.05), 99% less than the superimposed pressure change observed in lenses without TRPV1 inhibition (Fig. 5D, gray circles).

Hydrostatic Pressure Changes Induced by Ciliary Muscle Contraction and Relaxation Were Propagated Throughout the Lens

To determine if the changes in hydrostatic pressure measured in cells near the lens surface were propagated to the center of the lens, we measured the hydrostatic pressure gradient across the entire lens in untreated control (open diamonds), tropicamide-treated (filled squares), or pilocarpine-treated (filled circles) lenses (Fig. 6). Pressures were measured at four or five locations in individual lenses with or without drugs, and data from all lenses in each group were pooled and plotted against the normalized distance from the lens center. Fitted curves represent an equation that relates intercellular water flow through gap junctions to the hydrostatic pressure gradient.20 In control lenses, the best-fit value for the average pressure at the lens center was 5/8 mm Hg, in good agreement with previously published values.17,20,26 In lenses pretreated for 30 minutes with 0.1% tropicamide, the best-fit pressure value at the lens center was 262 mm Hg, a reduction of 24%. In lenses where the ciliary muscle was contracted with pilocarpine for 30 minutes, the predicted central pressure was elevated to 496 mm Hg (the data points stop midway through the lens because the column of mercury on our manometer ends at 400 mm). Pilocarpine-treated lenses also had a predicted positive pressure in the lens surface cells of 13 mm Hg.

The p110α Catalytic Subunit of PI3K Was Required for Pilocarpine To Increase Lens Hydrostatic Pressure

Previous work had suggested that the inhibitory effect on the Na+/K+/2Cl− ATPase mediated by TRPV1 was dependent on the activation of PI3K signaling.12,17 Contraction of ciliary muscles in eyes with intact zonules from knockout mice lacking the p110α catalytic subunit of PI3K failed to result in increased lens intracellular hydrostatic pressure. PI3K knockout lenses had stable initial pressure values of 22 ± 5 mm Hg that did not increase after the addition of pilocarpine to the bath (Fig. 7A). On average, application of pilocarpine to the PI3K knockout lenses raised the hydrostatic pressure near the surface by only 0.2 ± 0.9 mm Hg after 100 minutes (Fig. 7B, white hexagons, P > 0.05), significantly less than the superimposed pressure change induced in wild-type lenses (Fig. 7B, gray circles).

Contraction of the Ciliary Muscle With Pilocarpine Increased Phosphorylation of Akt in Lens Epithelial Cells

The PI3K-dependent inhibitory effect on Na+/K+/2Cl− ATPase activity was previously shown to be mediated through phosphorylation of its downstream effector Akt.12,17 To examine if Akt was involved following ciliary contraction, we dissected epithelial cell explants and stained them with a phospho-Akt antibody. Lens epithelial cells from wild-type mice that were not treated with pilocarpine showed low levels of phospho-Akt staining (Figs. 8A–C, n = 4). In mice treated with 0.2% pilocarpine, elevated staining of phospho-Akt was observed at 10 minutes (Figs. 8D–F, n = 4) and 20 minutes.
Figs. 8G–I, n = 4) after drug application, the same time frame when hydrostatic pressure is increasing. We have previously shown that lens-specific knockout of the p110α catalytic subunit of PI3K, or its opposing phosphatase PTEN, resulted in significantly decreased or increased levels of phospho-Akt, respectively.17,25 To confirm the specificity of our phospho-Akt antibody, we stained lens epithelia from p110α knockout mice and found that they lacked phospho-Akt staining (Fig. 8J, n = 2). In contrast, lens epithelial cells from PTEN knockout mice had elevated levels of phospho-Akt staining (Fig. 8K, n = 2).

**DISCUSSION**

We have examined whether forces exerted on the lens by the ciliary muscle through the zonules of Zinn affected the regulation of lens fluid circulation. We showed that the circumference of the ciliary body increased following relaxation with tropicamide, and decreased following contraction with pilocarpine, consistent with the idea that ciliary muscle modulation leads to stretching or relaxing of the zonular fibers, which in turn would affect zonular tension on the lens. Relaxation of the ciliary muscle caused a reduction in lens hydrostatic pressure that was blocked by inhibition of TRPV4 and depended on intact zonules. Ciliary contraction increased lens intracellular pressure in a zonule-dependent manner that was blocked by inhibition of TRPV1, or deletion of the p110α catalytic subunit of PI3K. These data illustrated that a previously described feedback control system for lens intracellular hydrostatic pressure is dynamically modulated by tension exerted by the ciliary muscle through the zonules of Zinn.

Although the lens signaling cascades activated by ciliary muscle contraction/relaxation appear to include some of those...
identified in regulation of the surface intracellular hydrostatic pressure, there are important differences in the responses. The previously described control system for hydrostatic pressure maintained zero pressure at the lens surface. When TRPV1 was pharmacologically activated, the surface hydrostatic pressure briefly became positive, but this was sensed by TRPV4, which was activated to restore pressure to zero. In our current study, when the ciliary muscle was pharmacologically contracted, TRPV1 was activated and caused a positive pressure change that propagated to the center of the lens.

**FIGURE 5.** Intact zonules and TRPV1 activity were required for pilocarpine to increase lens hydrostatic pressure. (A) Intracellular pressures in lenses without intact zonules (○) were unaffected by the addition of 0.2% pilocarpine to the bath. (B) Application of pilocarpine to free lenses increased the hydrostatic pressure near the surface by a mean value of 1.3 ± 2.2 mm Hg after 100 minutes (P > 0.05, n = 6). (C) When lenses with intact zonules were preincubated in the TRPV1 inhibitor A-889425 (▼), the subsequent addition of pilocarpine had no effect on pressure. (D) Application of pilocarpine to the TRPV1-inhibited lenses raised the mean hydrostatic pressure by 0.2 ± 1.2 mm Hg after 100 minutes (P > 0.05, n = 4). The pressure increase observed in lenses with intact zonules and uninhibited TRPV1 activity (●) is shown for comparison in (B, D).

**FIGURE 6.** The hydrostatic pressure gradient across the entire lens was modulated by tropicamide and pilocarpine. The hydrostatic pressure gradient was determined in the absence of drugs (○) or the presence of 0.1% tropicamide (●) or 0.2% pilocarpine (●) and plotted against the normalized distance from the lens center. Solid lines are fits to an equation that relates intercellular water flow through gap junctions to the hydrostatic pressure gradient.

**FIGURE 7.** PI3K signaling was required for pilocarpine to increase lens hydrostatic pressure. (A) Application of 0.2% pilocarpine to lenses with intact zonules from knockout mice lacking the p110α catalytic subunit of PI3K (▲) failed to increase intracellular hydrostatic pressure. (B) Application of pilocarpine to the PI3K knockout lenses raised the hydrostatic pressure by 0.2 ± 0.9 mm Hg after 100 minutes (P > 0.05, n = 4). The pressure increase observed in wild-type lenses with intact zonules (●) is shown for comparison in (B).
FIGURE 8. Lens epithelial cells showed increased Akt phosphorylation after ciliary muscle contraction with pilocarpine. (A–C) Lens capsules with adherent epithelial cells from untreated wild-type lenses had low levels of phospho-Akt. (D–F) Ten minutes after 0.2% pilocarpine treatment, epithelial phospho-Akt staining was notably increased (G–I) and remained high 20 minutes after pilocarpine was applied. (J) Lens epithelia from p110α knockout mice lacked phospho-Akt staining. (K) Lens epithelia from PTEN knockout mice had high basal levels of phospho-Akt staining.
This did not appear to activate TRPV4, as the positive pressure change persisted for as long as the muscle was contracted, and included a small increase in surface pressure. Similarly, in the previous study, when TRPV4 was pharmacologically activated, the surface hydrostatic pressure briefly became negative, but this was sensed by TRPV1, which was activated to restore surface pressure to zero. In our current work, when the ciliary muscle was pharmacologically contracted, TRPV4 was activated and caused a drop in intracellular pressure that propagated to the lens center. This did not appear to activate TRPV1, as the negative pressure change persisted for as long as the muscle was contracted, though the surface pressure remained zero. These observations suggest an additional control system that uses some of the components of the system that regulates surface hydrostatic pressure. In addition, this new system seems to target the magnitude of the entire center-to-surface gradient in intracellular hydrostatic pressure rather than maintaining the surface hydrostatic pressure at 0 mm Hg.

Lens epithelial cells have been shown to express muscarinic M1 receptors. These are not likely to be affected by tropicamide, as it is a muscarinic M4 receptor-prefering antagonist. However, pilocarpine activates M1 receptors, which could potentially influence the changes in lens hydrostatic pressure that we observed. There are at least two arguments against this idea. First, pilocarpine was shown to produce a Ca2+-ATPase with ouabain caused the release of water bound to crystallin proteins in the lens center, and this change in protein hydration produced the observed increase in refractive index in this area of the lens. It has been previously shown that exposure of lenses to external pressure changed the state of water bound to protein, supporting the idea that changes to the internal pressure of the lens generated by sodium transport could alter the water content in the lens. The pressure changes we have observed following ciliary contraction or relaxation occur on a much slower time course than the physical process of accommodation, making them unlikely to participate in dynamic focusing in younger lenses. However, in older lenses, ongoing stimulation of TRPV1 and TRPV4 by the zonules could induce lens pressure changes resulting in altered water distribution that contributes to some of the more slowly developing age-specific pathologies of the lens like presbyopia or cataract.

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