AQUAPORIN-1 MEDIATED FLUID MOVEMENT IN OCULAR TISSUES

by

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DEDICATION

This work is dedicated to my beautiful wife Kristine. I look to her for strength and reassurance on a daily basis and without fail she is always there for me. It has been a great fortune to spend so much of my life already with my best friend and I look forward to spending the rest of my days with her. Kristine, I love you very much and I thank you. Ella, may you follow in your mother's footsteps, then you can do no wrong.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Aquaporin proteins significantly increase water permeability across tissues and cell membranes. Ocular tissues, including the trabecular meshwork (TM) and retinal pigment epithelium (RPE), are especially reliant on aquaporin mediated water movement for ocular homeostasis. Even though bulk fluid movement is paracellular through the TM and transcellular through the RPE, both express aquaporin-1 (AQP1). The role and regulation of AQP1 as it relates to homeostasis in different ocular tissues is not well understood. I hypothesized that ocular tissues respond to external mechanical and molecular cues by altering AQP1 expression and function in order to regulate ocular fluid movement and maintain homeostasis.

To test how AQP1 function is altered in response to external cues in order to maintain tissue-specific homeostasis, I addressed the following two aims. The first aim was directed at determining how mechanical strain, an external stimulus that routinely affects TM function, influences AQP1 expression and TM homeostasis. Primary cultures of human TM were subjected to static and cyclic stretch and then analyzed for changes in AQP1 expression by western blot and cell damage by activity of lactate dehydrogense (LDH) in conditioned media. The results show AQP1 expression and LDH release significantly increased with static stretch. Analysis of LDH release with respect to AQP1 expression revealed an inverse linear relationship ($r^2 = 0.7780$).

The second aim was directed at characterizing signaling mechanisms responsible for regulating fluid transport in RPE, previously shown to be dependent upon AQP1. I treated primary cultures of human RPE with either atrial natriuretic peptide (ANP) or 8-

bromo-cyclic guanosine monophosphate (8-Br-cGMP) in the presence or absence of Anantin (ANP-receptor inhibitor) or H-8 (Protein Kinase G inhibitor). The results show that ANP and 8-Br-cGMP significantly increased apical to basal net fluid movement ($p \leq 0.05$, $n = 3$). Inhibition of these effects was successful with Anantin treatment but not with application of H-8.

The data presented demonstrate a novel role of protection for AQP1 in TM, and also expand upon cGMP dependent regulation of RPE fluid transport. The combined studies indicate tissue specific AQP1 regulation may offer new avenues to target water movement in treatment of ocular pathologies.

CHAPTER 1

INTRODUCTION, HYPOTHESIS, AND AIMS

1.1 General Introduction

Water movement is a critical part of homeostasis in tissues throughout the human body. From blood flow to metabolism, processes throughout the body are dependent upon efficient transfer of water. Disruption of water movement has been associated with pathological conditions in tissues ranging from the kidney to the brain. Of particular interest are ocular pathologies associated with disrupted fluid movement, such as glaucoma and retinal detachment, both of which can result in loss of vision (Kang and Luff, 2008, Quigley, 1996).

Ocular tissues are dynamic and require regular fluid movement to support basic functions. Aqueous humor drainage through the trabecular meshwork (TM) is important in removal of harmful byproducts (Liton and Gonzalez, 2008) from the anterior segment and regulation of intraocular pressure. The balance between aqueous inflow and outflow mechanisms, as well as response of tissues such as the TM to these processes, can have dramatic effects on intraocular pressure (Johnson, 2006), a characteristic closely monitored in glaucoma patients. In the posterior of the eye, the retinal pigment epithelium (RPE) is responsible for the absorption of solutes and water from the subretinal space into the blood (Steinberg, 1979). Tight regulation of the composition in the subretinal space is important for proper photoreceptor function and water movement across the RPE is a critical component of retinal attachment.

Aquaporins (AQP) are a key component to mediating ocular fluid movement. Six known aquaporins are expressed in the eye across a variety of tissue types suggesting subtle differences between the channels in terms of regulation and function. Aquaporins in the eye include AQP0, 1, 3, 4, 5, and 9. Aquaporin expression is detectable from the anterior segment in the lens (Gorin, et al., 1984, Stamer, et al., 1994, Zampighi, et al., 2002) and conventional outflow pathway (Hamann, et al., 1998, Stamer, et al., 1995) to the posterior segment in the retina and RPE (Kang, 2005, Stamer, et al., 2003). Aquaporin-1 is found in the NPE, the human lens epithelium, Schlemm's canal (SC), trabecular meshwork (TM), and the retinal pigment epithelium (RPE).

Aquaporin-1 is primarily a constitutively active water channel though previous studies have indicated modes of regulation for the channels. Induction of AQP1 expression has been observed with hypertonic treatment of cells and tissues (Leitch, et al., 2001, Umenishi, et al., 2004, Umenishi and Schrier, 2002). Phosphorylation and permeability studies following treatments with secretin and kinase activators support the potential for posttranslational regulatory mechanisms of AQP1 function (Han and Patil, 2000, Patil, et al., 1997). Furthermore, AQP1 has been implicated in cation conductance dependent upon cyclic nucleotide binding (Anthony, et al., 2000). There are other precedents for aquaporin regulation and variability of function, such as AQP2 phosphorylation (Brown, 2003), AQP6 conductance of anions (Hazama, et al., 2002, Yasui, et al., 1999), and AQP4 expression regulated by mechanical stress in skeletal muscle tissue (Frigeri, et al., 2004). Collectively, these data indicate aquaporin regulation and function is variable between aquaporins and tissues.

The TM and RPE are two ocular tissues that share expression of AQP1, but the primary pathway of fluid movement through each of these tissues is different. The TM is responsible for regulating a paracellular passage of fluid through the conventional outflow pathway. Previous studies have demonstrated however, that AQP1 is not necessary for bulk fluid flow suggesting an alternative role for the water channel. Conversely, the presence of AQP1 in the RPE has been shown to be important for transcellular fluid movement. Interestingly, the RPE shares characteristics with the choroid plexus where AQP1 function has been shown to include a cation conductance when stimulated with cGMP indicating additional functions and regulatory mechanisms may be in place for AQP1 function in the RPE (Boassa, et al., 2006). While fluid movement in each of these tissues may serve a different purpose and be regulated through different means, they each express AQP1 suggesting multiple roles for this protein. These combined pieces of evidence indicate a potential tissue-specific role for AQP1 in ocular tissues, responsive to a surrounding microenvironment in order to maintain homeostasis.

1.2 Central Hypothesis:

Ocular tissues respond to external mechanical and molecular cues by altering aquaporin-1 expression or function in order to regulate ocular fluid movement and maintain homeostasis.

1.3 Specific Aims: To test the central hypothesis I constructed the following aims.

Specific Aim 1 – To determine the role of aquaporin-1 in trabecular meshwork response to mechanical strain. The rationale for the first aim was that the TM is continuously subjected to mechanical strain that affects the integrity and viability of the TM. The *working hypothesis* for this aim was; Aquaporin-1 contributes in trabecular meshwork homeostatic response to dynamic mechanical strain. The information in chapter 3 indicated aquaporin-1 expression was regulated in response to mechanical strain and that aquaporin-1 expression was important for cell viability during times of mechanical strain.

Specific Aim 2 – To determine the contribution of aquaporin-1 to fluid movement in retinal pigment epithelium. The rationale for the second aim was that AQP1 has been shown to contribute to fluid movement in the choroid plexus as a cation channel as well as a water channel following stimulation with atrial natriuretic peptide (ANP). The choroid plexus and the RPE are unique in the apical localization of the Na^+/K^+ ATPase. The *working hypothesis* for this aim was; Atrial natriuretic peptide stimulates fluid transport in retinal pigment epithelium. The information in Chapter 4 suggests fluid

movement across retinal pigment epithelium monolayers is stimulated by atrial natriuretic peptide via cyclic guanosine monophosphate signaling.

CHAPTER 2

CRITICAL LITERATURE REVIEW[1]

2.1 Aquaporins Discovery, Structure, and Function

2.1.1 Water Permeability and Aquaporin Discovery

Water comprises approximately 65-70% of the human body and is important for processes in all tissues. In order for proper operation of tissues, water must move selectively across the tissues and membranes. Biological barriers are permeated by water due to the presence of water channels that facilitate water transport.

Though the isolation of the first aquaporin did not occur for some time, a number of early studies provided preliminary support for the presence of proteins that behave as water permeable channels. The permeability of red blood cells in comparison with lipid bilayers using tritiated water showed permeability of the red blood cells was greater indicating another mode of transport outside of simple diffusion was present in red blood cells that was not available in lipid bilayers (Paganelli and Solomon, 1957). Inhibition of red blood cell water permeability was later accomplished using sulfhydryl reagents suggesting the presence of disulfide bonds for the reagent to bind (Macey and Farmer, 1970). Estimation of the pore size for water permeability in red blood cells was achieved by creating similar pores in lipid bilayers using antibiotics and comparing the hydraulic conductivity of the lipid bilayers and red blood cells (Solomon and Gary-Bobo, 1972).

[1] Portions of this chapter were previously published in WD Stamer, NW Baetz and AJ Yool. Ocular aquaporins and aqueous humor dynamics. The Eye's Aqueous Humor in Current Topics in Membranes. Volume Editor: MM Civan 2009.

Early attempts at characterizing and identifying the protein responsible for red blood cell water permeability included application of radiolabeled sulfhydryl reagents to cell membranes that showed a localization of the radiolabel with Band 3, later termed anion exchanger 1 (Brown, et al., 1975). Analysis of the amino acid sequence indicated the protein was a member of the major intrinsic protein (MIP) family (Gorin, et al., 1984). Collectively, the evidence supported the possibility of a water channel.

In 1987, a 28 kilodalton protein was isolated from red blood cells (Agre, et al., 1987) initially labeled CHIP28 or "channel like integral protein." Studies by Smith and Agre demonstrated the protein was most likely a homotetramer wherein one out of four subunits was glycosylated (Smith and Agre, 1991). In order to determine the function of this protein, Preston and Agre (Preston and Agre, 1991) cloned the gene from cDNA libraries from red blood cells and expressed the protein in *Xenopus* oocytes subjected to hypotonic saline (Preston, et al., 1992). The resultant swelling of the oocytes provided evidence that the newly discovered proteins were water channels, ultimately renamed aquaporins.

Aquaporins provide molecular pathways for the movement of water and selected small solutes across cell membranes (King, et al., 2004). Aquaporins are found throughout the kingdoms of life, including prokaryotes and eukaryotes. In mammals, there are at least 12 classes of aquaporins (AQP0 to AQP11), which show tissue-specific patterns of expression. These channels are broadly classified as orthodox aquaporins, selective for water, and the aquaglyceroporins including AQP3, AQP7, and AQP9, that allow transmembrane movement of glycerol as well as water. Beyond the simple

bimodal classification scheme, our understanding of permeability properties is being extended steadily to include roles for aquaporins in the transport of other compounds including ions, gases, and small organic compounds, as reviewed (Yool, 2004). Much remains to be discovered about the full range of functional properties of this family of channels.

2.1.2 Aquaporins in Mammalian Tissues and Associated Pathologies

Aquaporin proteins are found throughout various tissues in mammals and knockout models have demonstrated the importance of these channels. Common examples of tissues that express aquaporin proteins include the kidney, lung, and the brain. Studies of the kidney have revealed at least six aquaporins present. Aquaporins 2, 3, 4, (Knepper, et al., 1996) are each expressed in the collecting duct, while aquaporins 1, 7, and 8 have been shown in the proximal tubules (King and Yasui, 2002). Knockout mice for AQP2 have shown a reduced urinary concentrating effect indicating aquaporins are important in routine kidney function (Ma, et al., 1998). Aquaporins in the lung include AQP1, 3, 4, and 5 (Borok and Verkman, 2002). While AQP1 is primarily expressed in the vasculture, AQP3 and 4 are found in epithelial cells and AQP5 is found predominantly in the Type I alveolar cells of the lung (Borok and Verkman, 2002). Knockout mice for AQP5 have shown a decreased capacity for fluid secretion in the airways and significant reductions in water permeability in the lungs (Ma, et al., 2000). Aquaporin 4 is most notably found within astroglial cells of the brain (Nielsen, et al., 1997, Rash, et al., 1998) and AQP1 is found in the choroid plexus epithelium (Bondy, et

al., 1993, Speake, et al., 2003), responsible for secretion of cerebrospinal fluid. Knockout models of AQP4 have demonstrated decreased brain edema after insult with meningitis (Papadopoulos and Verkman, 2005). Interestingly, the eye has a multitude of aquaporin proteins present in various ocular tissues with a range of functions which will be discussed in following sections.

2.1.3 Aquaporin-1 Structure and Function

Crystal structural data available for AQP1 has verified classic work in the field that first defined general principles of structure in the archetypal member of this family of proteins (de Groot, et al., 2003, Jung, et al., 1994, Ren, et al., 2001, Sui, et al., 2001). Aquaporins are tetrameric complexes of subunits (Fig. 2.1). Each subunit has six full transmembrane domains per subunit, intracellular N and C terminal domains, and water pores framed by loops B and E. The hourglass model of a subunit of AQP1 was envisioned as a narrow pore pathway within each subunit, with the hallmark asparagine-proline-alanine (NPA) motifs located near the center of the membrane interior at the junction of the folded B and E loops (Jung, et al., 1994). In the intrasubunit pores, the orthodox aquaporins show a high selectivity for water, excluding solutes, ions, and protons. The central pore at the fourfold axis of symmetry in the tetramer may provide a parallel pathway for regulated movement of other molecules, such as CO_2 and ions, in specialized subsets of aquaporins (Yu, et al., 2006). For example, CO_2 permeation through AQP1 could serve a physiological role in membranes that have a low intrinsic permeability to the gas. Based on analysis of free energy barriers, the AQP1 central

cavity is favored over the monomeric channel as a candidate pathway for CO_2 (Hub and de Groot, 2006). Molecular dynamics simulations suggest the central pore is a pathway for cations in AQP1 (Yu, et al., 2006). It is possible that the two AQP1 channel states exist as alternatives, with Na^+ permeating the hydrated central pore in the hypothetical ‘‘open’’ state, and CO_2 moving through the dehydrated pore in a ‘‘closed’’ state. These multifunctional properties add complexity to the potential roles of these channels in tissues such as the eye.

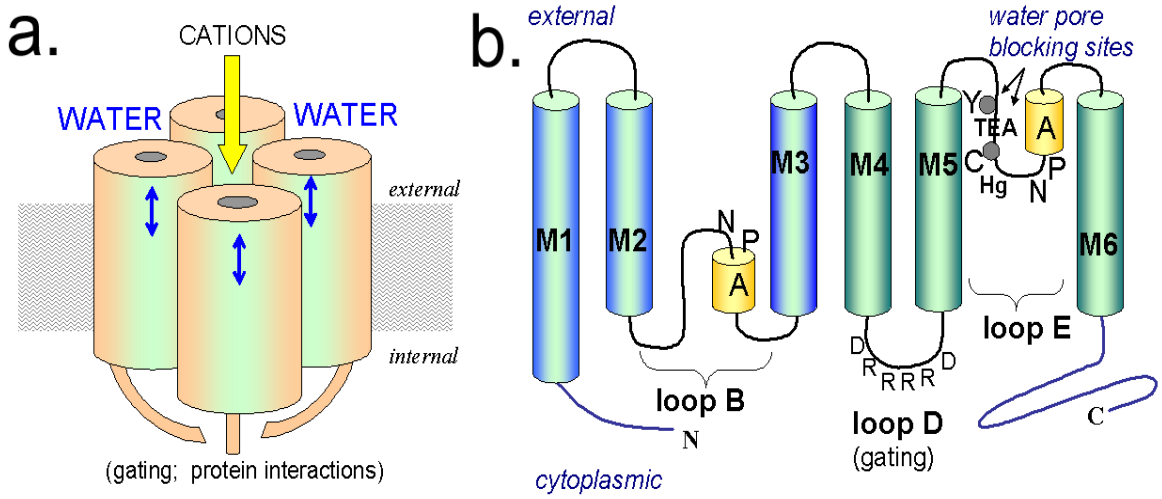

Figure 2.1: Tetrameric organization and subunit transmembrane topology of the aquaporin-1 protein. (A). Aquaporins are assembled as homomeric assemblies of four subunits. The constitutive water-selective pores are located within each subunit, and for AQP1 the proposed ion channel is located in the center of the tetrameric complex (Yu, et al., 2006). (B) Diagram of main features of the transmembrane topology of a human AQP1 subunit, indicating loops A to E and six full transmembrane regions M1 to M6. Selected functional domains include the proposed gating region (loop D), with arginines (R159 and R160) suggested to serve in the cGMP-induced activation of the AQP1 ionic conductance, and the asparagine-proline-alanine (NPA) motifs in loops B and E that contribute to water-selective pore structures. Tyrosine (Y187) and cysteine (C189) in loop E have been shown to mediate block of water permeability by extracellular tetraethylammonium and mercuric compounds, respectively. The carboxyl terminal domain contains regions that influence cGMP-induced activation, enable protein-protein interactions, and in addition to other intracellular protein domains of AQP1 might be sites of modulation.

2.2 Ocular Distribution of Aquaporins

2.2.1 Ocular Aquaporins

As is the kidney, the eye is a water-transporting organ. The eye rivals the kidney in terms of the number of aquaporin homologues that are expressed, and surpasses the kidney in terms of the number of different cell types that express aquaporin channels. To date, the selective expression of 6 different aquaporin homologues in 8 different cell types located in 10 different ocular tissues have been described (Table 2.1). Thus, the distribution of AQP0, AQP1, AQP3, AQP4, AQP5, and AQP9 are for the most part nonoverlapping and found in epithelial cells, endothelial cells, fibroblasts, trabecular meshwork (TM) cells, lens fiber cells, neuronal, glial, and photoreceptors in the eye. These aquaporin expressing cells populate the cornea, conjunctiva, lens, iris, TM, ciliary body, sclera, retina, choroid, and optic nerve. Aquaporin-0 is found primarily in the lens fiber cells of the crystalline lens, but has been recently detected in testis and liver as well (Tietz, 2005).

2.2.2 Lens Aquaporins

The clarity of the organic lenses of the eye is highly dependent upon water homeostasis, and thus upon aquaporin channel function. The crystalline lens expresses two aquaporins, AQP1 in the monolayer of epithelial cells, which covers the anterior surface, and AQP0 in the terminally differentiated lens fiber cells, which forms the bulk of the lens' mass. AQP0 constitutes almost half of the total protein at the plasma membrane of lens fiber cells. AQP0 was the first aquaporin discovered (Gorin, et al.,

1984) however, its role as a water channel was not completely understood until later because of its low capacity for water subject to physiological regulation (Chandy, et al., 1997, Mulders, et al., 1995). For example, changes in intracellular signals in the lens, Ca^{2+}calmodulin and pH, regulate the water permeability of endogenously expressed AQP0 in lens fiber cells, but not that of AQP1 natively expressed in lens epithelial cells (Varadaraj, et al., 2005).

In addition to a role as a regulated water channel, AQP0 is thought to have a structural function as a cell–cell adhesion protein (Mulders, et al., 1995). In the lens, microdomains located at the junctions between fiber cells form two dimensional arrays of AQP0 proteins that are thought to provide cell–cell adhesion, and are surrounded by densely packed gap junction channels that mediate intercellular communication (Buzhynskyy, et al., 2007, Zampighi, et al., 2002). AQP0 arrays appear to be stabilized by physical associations with both gap junction proteins and the lens-specific intermediate filament proteins filensin and CP49 (Lindsey Rose, et al., 2006, Yu, et al., 2005).

Consistent with its role in maintaining lens clarity, mutations in AQP0 result in cataract (Shiels and Bassnett, 1996). Dominantly inherited cataracts were found in two families carrying different point mutations in the gene for AQP0, presenting different clinical features: the mutation E134G associates with a unilamellar cataract, whereas the mutation T138R correlates with multifocal opacities that increase with age (Francis, et al., 2000). Coexpression of mutant AQP0 with wild type in *Xenopus oocytes* decreases water permeability, and high levels of coexpression of the mutant impairs regulation of

wild-type water fluxes by Ca^{2+}. These findings suggest that the regulated water permeability of AQP0 could be an important component in lens homeostasis and development (Kalman, et al., 2006). Taken together, accumulating evidence suggests that AQP0 is more than a physical anchoring structure, but also serves a role in the movement of fluids within the lens, with details of its functional roles yet to be defined.

Maintenance of lens transparency depends not only on AQP0, but also on AQP1. The role of the high capacity AQP1 channels in the lens epithelium is likely to be a more orthodox one that is, to facilitate the efficient movement of water across its epithelial surface that will contribute to water circulation in the lens, and thus to lens health and transparency. For instance, osmotic water permeability was decreased almost 3-fold in epithelial cells of intact lenses from AQP1-deficient mice as compared to wild type, and the loss of lens transparency was accelerated more than 50-fold during osmotic stress (Ruiz-Ederra and Verkman, 2006).

Table 2.1: Summary of aquaporin expression in the human eye

Tissue	Cell Type	Aquaporin	References
Cornea	Epithelia	AQP3, AQP5	(Funaki, et al., 1998, Hamann, et al., 1998, Patil, et al., 1997, Raina, et al., 1995)
	Keratocytes	AQP1	(Hamann, et al., 1998)
	Endothelia	AQP1	(Echevarria, et al., 1993, Hamann, et al., 1998, Hasegawa, et al., 1994, Hasegawa, et al., 1993, Nielsen, et al., 1993, Patil, et al., 1997, Stamer, et al., 1994)
Conjunctiva	Epithelia	AQP3, AQP5	(Frigeri, et al., 1995, Hamann, et al., 1998, Oen, et al., 2006)
Lens	Epithelia	AQP1	(Hamann, et al., 1998, Hasegawa, et al., 1994, Nielsen, et al., 1993, Patil, et al., 1997, Stamer, et al., 1994)
	Fiber cells	AQP0	(Broekhuyse, et al., 1979, Fitzgerald, et al., 1983, Gorin, et al., 1984, Zampighi, et al., 1989)
Iris	Anterior and Posterior Epithelia	AQP1	(Hamann, et al., 1998, Hasegawa, et al., 1994, Nielsen, et al., 1993, Patil, et al., 1997, Stamer, et al., 1994)
Conventional Outflow Tract	Trabecular meshwork	AQP1	(Stamer, et al., 2001, Stamer, et al., 1995, Stamer, et al., 1994)
	Schlemm's Canal	AQP1	(Hamann, et al., 1998, Stamer, et al., 1994)
Ciliary Body	Non-pigmented epithelia	AQP1, AQP4	(Frigeri, et al., 1995, Hamann, et al., 1998, Hasegawa, et al., 1994, Hasegawa, et al., 1993, Nielsen, et al., 1993, Patil, et al., 1997, Stamer, et al., 1994)
Sclera	Fibroblasts	AQP1	(Hamann, et al., 1998)
Retina	Muller	AQP1, AQP4	(Frigeri, et al., 1995, Hamann, et al., 1998, Kim, et al., 1998, Nagelhus, et al., 1998)
	Retinal pigment epithelia	AQP1	(Stamer, et al., 2003)
	Amacrine	AQP1, AQP9	(Iandiev, et al., 2006, Kang, et al., 2005, Kim, et al., 2002, Kim, et al., 1998)
	Photoreceptors	AQP1	(Iandiev, et al., 2006, Nagelhus, et al., 1998)
Optic Nerve	Astrocytes	AQP4	(Nagelhus, et al., 1998)
Capillaries	Endothelial	AQP1	(Hamann, et al., 1998)

2.2.3 Corneal and Conjunctival Aquaporins

The selective expression of AQP1, AQP3, AQP4, and AQP5 in distinct ocular epithelia compels an expectation that each aquaporin class has a distinct and specific role in complex regulation of water movements in the eye. At the anterior surface of the mammalian eye, both the corneal and conjunctival epithelia express two aquaporins, AQP3 and AQP5. In contrast, a single monolayer of endothelial cells at the posterior surface of the cornea expresses AQP1 channels and interface with aqueous humor in the anterior chamber. Here, AQP1 is thought to function in facilitating the efficient transport of water out of the corneal stroma and into the anterior chamber to help maintain clarity. Evidence of the role of aquaporin in maintaining corneal hydration, and thus clarity, was recently provided in aquaporin knockout mice. Corneal thickness was significantly decreased in AQP1 null mice and increased in AQP5 null mice (Thiagarajah and Verkman, 2002). While corneal transparency was not impaired under baseline conditions, the rate of corneal swelling was compromised in both AQP1 and AQP5 null mice when challenged upon exposure with hypotonic medium.

2.2.4 Ocular Aquaporins of Undetermined Function

AQP1 is located in several ocular tissues where its function is unclear. For example, AQP1 is localized to the apical and basolateral membranes of pigmented posterior epithelial and anterior myoepithelial cells of the iris (Table 2.1). The precise role of AQP1 in iris function is unknown, but may relate to changes in rapid water movement or cell volume that may occur upon contraction or relaxation during mydriasis

or miosis, respectively. AQP1 is also found in fenestrated and non-fenestrated capillaries in ocular tissues that include the choroid, ciliary body, sclera, and iris. As in other capillary beds of the body, particularly fenestrated, their functional role is uncertain. Finally, AQP1 is highly expressed by resident fibroblasts of the sclera and corneal stroma (keratocytes). Unfortunately, the role of AQP1 in fibroblast function both in the eye and elsewhere still needs to be determined (Gallardo, et al., 2002, Maeda, et al., 2005).

Aquaporin channels are expressed by cells responsible for the production and removal of aqueous humor from the eye. AQP1 and AQP4 localize to both the apical and basolateral plasma membranes of nonpigmented epithelial cells of the ciliary body, but are completely absent from pigmented epithelial cells (Fig. 2.2). In the ciliary processes, aquaporins function to enable formation of aqueous humor with the efficient passage of water, following salt transport, from the ciliary stroma into the posterior chamber (discussed in detail in the following section). After flowing between the lens and iris into the anterior chamber, the majority of aqueous humor exits the eye via the conventional (~70%) and unconventional (~25%) routes (Bill and Phillips, 1971), (Townsend and Brubaker, 1980), (Toris, et al., 1999). A small portion of water (~5%) travels posteriorly through the vitreous, and exits across the retina. AQP1 is expressed by cells that populate the conventional and posterior outflow routes. In the conventional outflow pathway, cells that cover the trabecular lamellae and occupy the juxtacanalicular region of the TM express AQP1 (Fig. 2.2). Additionally, endothelial cells that form part of the blood–aqueous barrier, Schlemm's canal (SC) endothelia, express AQP1 channels. The role of AQP1 in regulating aqueous movement through the conventional route is yet

uncertain (discussed in detail in the following section). A minor but significant amount of water exits the eye across a continuous monolayer of epithelial cells, the retinal pigment epithelium (RPE) that forms the blood–retina barrier and lies just posterior to the retina. In humans, AQP1 localizes to both the apical and basolateral membranes of RPE cells however, AQP1 has not been detected in RPE of other models such as mouse.

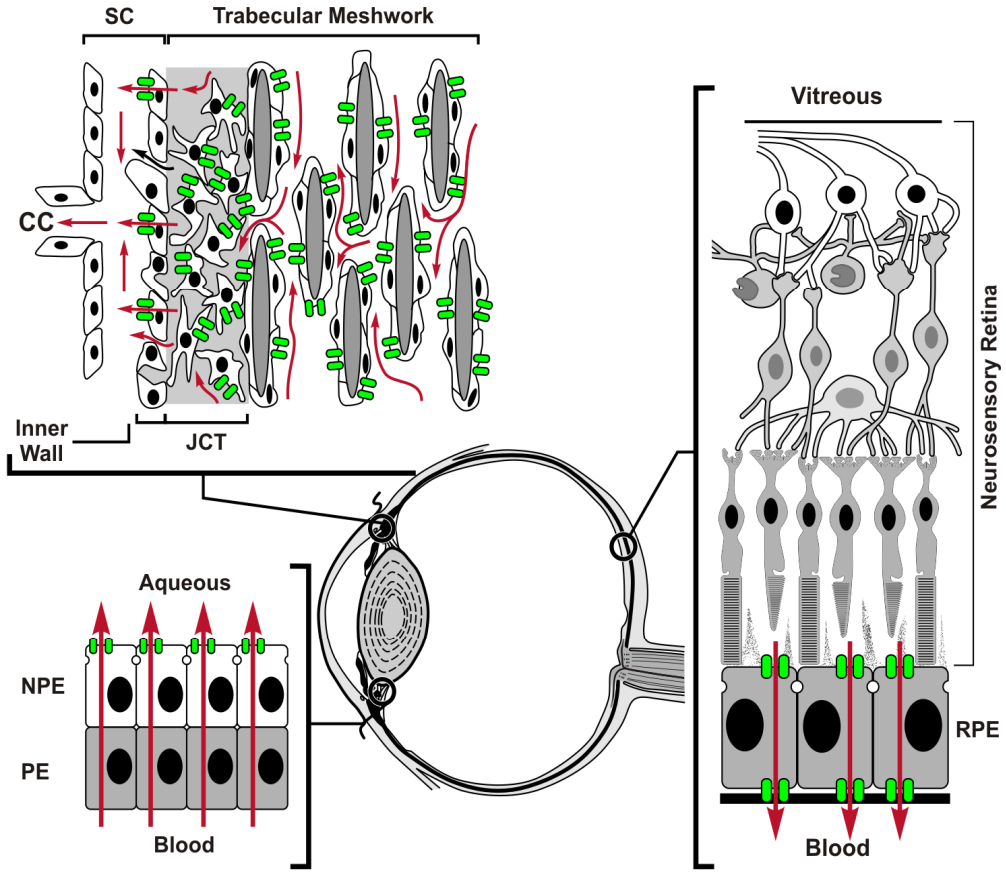

Figure 2.2: Distribution of aquaporin channels in ocular tissues that participate in aqueous humor dynamics. Shown is a schematic of the human eye and three ocular tissues in cross section; the conventional outflow pathway (top left), the ciliary epithelial bilayer (bottom left) and retinal pigment epithelium (right). Indicated in green are putative locations of aquaporin channels that participate in aqueous humor dynamics in these three tissues. Red arrows indicate direction of flow across/through these ocular tissues. SC=Schlemm's canal, JCT=juxtacanalicular tissue, CC=collector channel, NPE=nonpigmented epithelium, PE=pigmented epithelium, RPE=retinal pigment epithelium.

2.3 Ocular Fluid Movement

2.3.1 Aqueous Humor and Vitreous Humor

Vision is dependent upon the efficient movement of water between and within various structures of the eye. To facilitate the faithful transmission of light rays from the corneal surface to the retinal photoreceptors, the eye is pressurized, having three compartments that are filled with optically transparent fluids: Vitreous humor occupies ~80% of the interior volume of the eye and lies between the posterior face of the lens and the retina. Aqueous humor fills the other two compartments, the anterior and posterior chambers, located on either side of the iris. The circulation of aqueous humor from the posterior to the anterior chamber (and then out of the eye) enables the delivery of nutrients and removal of waste products from two specialized avascular tissues, the cornea and crystalline lens that function to focus light onto the retina. The clarity of these two organic lenses, and thus their ability to refract light, is exquisitely dependent upon water homeostasis within and the circulation of aqueous humor around their structures. For example, corneal clarity is reliant upon the maintenance of stromal water content by the cellular barriers that line either surface, while intraocular pressure is regulated within a narrow range by the balance of aqueous humor secretion and drainage. Not surprisingly, every tissue that produces, removes, or is in contact with aqueous humor contains aquaporins (AQPs) to facilitate the efficient and selective movement of water across ocular membranes.

2.3.2 Aqueous Humor Formation and Outflow

2.3.2.1 Aqueous Humor Formation

The rates of water movement across epithelial barriers in the renal tubular system and ciliary body are similar, among the highest measured in the body [0.6–1.2 cm^3/cm^2 (Brubaker, 1991)], and no doubt in part due to aquaporin expression (King, et al., 2004). In contrast to fluid resorption in renal tubules, secretion of aqueous humor by the ciliary epithelium occurs against both oncotic and hydrostatic gradients. To overcome these forces, water follows the active transport of salt across the two ciliary epithelia.

Aqueous humor formation is thought to involve a three step process by the epithelial bilayer that lines the processes of the ciliary body (Civan and Macknight, 2004). First, paired sodium-proton and chloride-bicarbonate antiporters play a major role in transferring sodium and chloride from the ciliary body stroma into pigmented epithelial cells. Sodium and chloride easily pass by simple diffusion from pigmented cells into the nonpigmented cells through gap junctions before they are actively moved to the posterior chamber via a combination of Na/K ATPases, Cl- channels, and Na/K/2Cl- cotransporters. Because gap junctions between nonpigmented and pigmented cells allow the free movement of water and salt, and pigmented cells do not contain tight junctions; aquaporin channels (AQP1 and AQP4) appear to be needed only in nonpigmented cells. Interestingly, even though tight junctions between nonpigmented epithelial cells form the blood–aqueous barrier and eliminate paracellular passage of solute and water, aquaporins localize to plasma membranes on both apical and basolateral sides; suggesting that water is drawn into nonpigmented cells both from pigmented cells, through gap junctions, and

from interstitial space on lateral sides, through aquaporins. Finally, water exits nonpigmented cells and enters the posterior chamber in part through AQP1 and AQP4 channels on the basal membranes.

The functional contribution of aquaporin channels to aqueous humor secretion in vivo was demonstrated in mice lacking AQP1, AQP4, or both (Zhang, et al., 2002). Despite probable compensatory mechanisms, intraocular pressure in the mice lacking aquaporins (AQP1, AQP4, or AQP1/AQP4) was significantly lower than their wild-type littermates (Fig. 2.3). Depression of intraocular pressure varied between 1 and 2 mm Hg, depending upon the strain of mice and the missing aquaporin homologue(s). This decreased intraocular pressure in mice lacking aquaporins was found to be in part due to lower levels of aqueous humor production. In these animals, aqueous humor production was measured using in vivo confocal microscopy after introduction of fluorescein into the anterior chamber. Figure 2.3 shows that fluorescein had a longer half-life in the anterior chamber of mice lacking one or both of the aquaporins expressed by the nonpigmented ciliary epithelium. These data in living animals agree with data obtained with cultured cells showing that transport of fluid across monolayers of nonpigmented epithelial cells is inhibited upon treatment with mercuric chloride (a potent blocker of AQP1 channels) and antisense oligonucleotides specific for AQP1 RNA (Patil, et al., 2001).

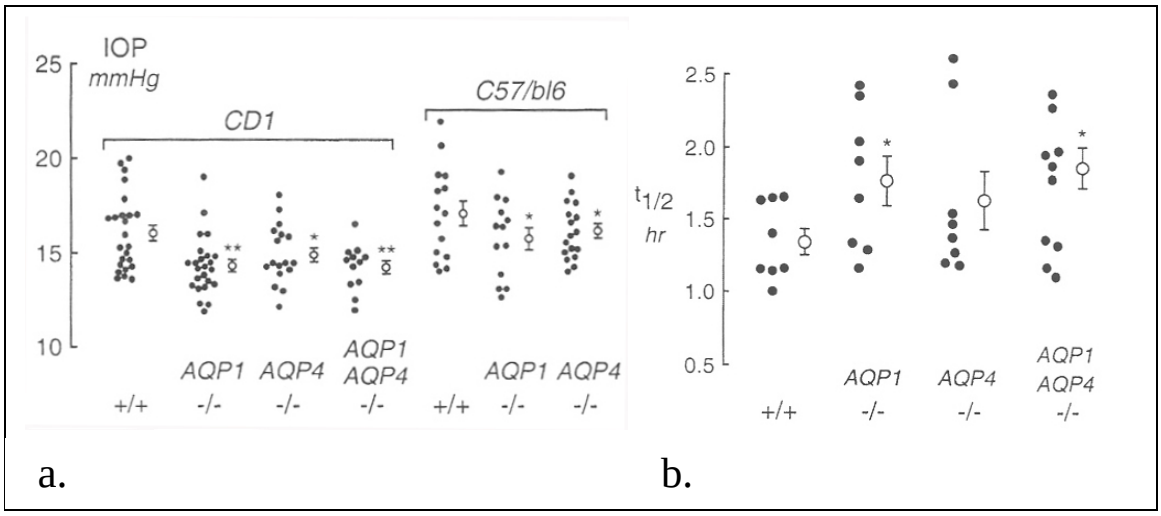

Figure 2.3: Intraocular pressure measurements and aqueous humor production in aquaporin null mice. Panel A shows results of IOP measurements in two different strains of wild-type mice, AQP1 and AQP4 null mice and AQP1/AQP4 double null mice. Shown are data from individual eyes (filled circles) and mean ±SE (open circles). *p<0.05, **p<0.002 (ANOVA). Panel B shows measurements of individual eyes (filled circles) and mean ±SE (open circles) for aqueous humor production in wild-type and AQP null mice. Data are expressed as half-times ($t_{1/2}$) for fluorescein disappearance. *p<0.05 (ANOVA). *Reprinted from Zhang et al., 2002*.

In addition to effects of altered aquaporin expression on membrane permeability, regulation of AQP1 and AQP4 by second-messenger systems was also shown to impact water movement across cell membranes. For example, phosphorylation of AQP1 by cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) was shown to increase fluid movement across cells heterologously expressing AQP1 by increasing AQP1 at the plasma membrane (Han and Patil, 2000). These data are consistent with known dependency of water movement across the ciliary epithelium upon intracellular levels of cAMP. However, the specific effects of AQP1 phosphorylation to cAMP-mediated changes in aqueous humor productions have not been demonstrated. With

respect to AQP4, phorbol ester treatment resulted in AQP4 phosphorylation and a consequential decrease in membrane permeability of cells heterologously expressing AQP4 (Han, et al., 1998). Interestingly, phorbol ester treatment of rabbit eyes decreased intraocular pressure by 40%, although a role for aquaporin involvement is unknown (Mittag, et al., 1987). Finally, atrial natriuretic peptide treatment of cells heterologously expressing AQP4 or AQP1 results in a decreased permeability to water (Patil, et al., 1997). Atrial natriuretic peptide is also known to inhibit secretion of aqueous humor and lower IOP; however, the role of aquaporins again remains to be defined (Crook and Chang, 1997, Fernandez-Durango, et al., 1999, Mittag, et al., 1987). In choroid plexus, a tissue that secretes cerebral spinal fluid and strongly expresses AQP1 channels, atrial natriuretic peptide similarly causes a decrease in fluid and salt transport; this process has been suggested to involve not only the water channel property of AQP1, but also the cyclic guanosine monophosphate-activated cationic conductance mediated by AQP1 (discussed in a following section; (Boassa, et al., 2006)). Part of the current study is aimed at determining if the ion channel property has any contribution to the regulatory effects of atrial natriuretic peptide signaling in the eye.

2.3.2.2 Aqueous Humor Outflow

2.3.2.2.1 Anterior Segment Outflow

While the role of aquaporins in aqueous humor inflow has been clearly demonstrated, the responsibility of aquaporins in outflow function is less certain. AQP1 is expressed abundantly in all regions of the conventional outflow tract, including the

inner (uveal and corneoscleral) and outer (juxtacanalicular tissue, JCT) TM and the inner wall of SC. However, outflow facility measurements in AQP1 null mice were not significantly different from their littermate controls (Zhang, et al., 2002). These results need to be interpreted with caution for several reasons. Since the conventional pathway regulates intraocular pressure by controlling the rate of aqueous humor drainage, there are likely multiple compensatory mechanisms to accommodate the loss of a single protein (AQP1 in this case). Next, effects of AQP1 deletion on outflow facility (hydrostatic-driven) may have been under the level of detection because hydrostatic-driven water permeability in other tissues is affected less by absence of AQP1 than is osmotic-driven water permeability (two-fold versus ten-fold); (Bai, et al., 1999). Additionally, appreciable differences in the anatomy and physiology of aqueous humor drainage exist between mice and humans. The TM is architecturally less complex in mice (composed of two to three layers of lamellae) than in humans (seven to eight layers). In mice conventional outflow accounts for roughly half of total outflow, whereas in humans conventional outflow accounts for about three-quarters of the total outflow. Thus, clinically relevant analyses of the specific contribution of AQP1 to conventional outflow would benefit from use of animal models that are carefully matched with key human parameters or by use of human tissue such as perfused anterior segments in organ culture (Johnson and Tschumper, 1987). With the organ culture model, AQP1 protein can be manipulated using gene transfer, gene silencing, or pharmacological blockers and effects on outflow facility can be monitored over time.

Indications about the potential role of AQP1 in the conventional outflow tract were provided using primary cells that were isolated from human donor eyes (Stamer, et al., 2001). In these experiments, AQP1 expression was manipulated using adenovirus vectors that carried AQP1 cDNA oriented in the sense or antisense direction. Interestingly, AQP1 overexpression was found to increase resting intracellular volume by ~9%, and thus decrease paracellular permeability of trabecular cell monolayers. The inverse occurred upon knockdown of AQP1 protein (by ~70%); where resting TM cell volume decreased by ~8%. These data were among the first to implicate a role for AQP1 in cell volume regulation in the outflow pathway. Since this report, several laboratories have shown that aquaporins often exist in protein complexes that appear to sense or regulate cell volume (Chan, et al., 2004, Krane, et al., 2001, Kuang, et al., 2004, Liu, et al., 2006).

In the conventional outflow tract, changes in volume of cells in the juxtacanalicular region or inner wall of SC have been shown to influence outflow facility [and thus intraocular pressure (Gual, et al., 1997)]. Volume changes in the JCT affect the geometry of the conventional tissues and impact flow pathways for aqueous humor. For example, a 10% decrease in cell volume results in ~25% increase in outflow facility (Al-Aswad, et al., 1999). At the inner wall of SC, changes in cell volume would be expected to impact transcellular (vs paracellular) routes for fluid. Such routes have been referred to as ‘‘border pores’’ (Ethier, et al., 1998). Since the inner wall of SC is the only continuous cell barrier that aqueous humor encounters before entering the systemic circulation, changes in the number of AQP1 channels at the cell surface would likely

affect the transcellular permeability of the barrier. The proportion of aqueous humor that utilizes transcellular versus paracellular routes presently is unknown, and thus the impact of aquaporin expression in SC cells on total outflow facility is uncertain (Ethier, 2002, Johnson, 2006).

A role for AQP1 in the JCT cells and SC cells can be envisioned in light of their contribution to outflow resistance (i.e., regulation of fluid transport out of the eye), but the function of AQP1 channels in TM cells that reside on the lamellar beams -presumably providing no appreciable resistance to flow due to the wide opening between beams- is unknown. One possibility is that AQP1 channels may accommodate rapid volume changes that could occur in the conventional outflow tract when the outflow tissues are subjected to mechanical deformation. Trabecular cells reside in a unique environment that is under continuous mechanical stress, both repetitive and intermittent (Ethier, 2002). For instance, during accommodation, the TM is stretched and forces are transmitted throughout conventional outflow tissues via tendons that originate in the ciliary muscle and attach to the basement membrane below the SC inner wall. In addition, conventional outflow tissues are continually perturbed due to the ocular pulse, blinking, squinting, or eye rubbing (Coleman and Trokel, 1969). Such everyday activities can rapidly and transiently elevate intraocular pressure by up to an order of magnitude (from 10 to 100 mm Hg). As tissues deform, the resident cells can be forced to change volume, and aquaporin could be playing a key role in allowing TM cells to change volume in the meshwork. Interestingly, in skeletal muscle a similar role for AQP4 has been hypothesized where aquaporins are thought to facilitate the rapid transfer of water from

blood to muscle during periods of intense activity, such as exercise (Frigeri, et al., 2004). If this hypothesis is true in the meshwork, the presence of AQP1 on uveal and corneoscleral meshwork cells emphasizes the dynamic biomechanical environment of the conventional outflow pathway.

2.3.2.2.2 Posterior Segment Outflow

A small but significant proportion of aqueous humor that is produced by the ciliary epithelia exits the eye posteriorly, across the retina and RPE. To facilitate this flux, there is a net apical to basolateral movement of solute across RPE cells (Marmorstein, 2001, Miller and Steinberg, 1977). In addition to active transport of solute, two passive mechanisms, intraocular pressure and oncotic pressure from the choroid, contribute to water movement across the RPE and into the choroid. Because the paracellular route is restricted by the presence of highly complex tight junctions that are essential to maintain the blood–retinal barrier [resistance 2000 Ohms*cm^2 (Joseph and Miller, 1991, Marmorstein, 2001)], water and solute must traverse cell membranes in a process likely to be facilitated by transporters and channels, including AQP1 channels.

The transport of solute across the RPE is dependent in part upon the concentration of potassium and sodium in the subretinal space (between the photoreceptors and RPE), which in turn is dependent upon ion conductances across photoreceptors during periods of light and darkness. During light onset for example, the subretinal potassium concentration decreases, causing changes in the activity of apically located potassium channels and transporters in the RPE that ultimately influence chloride transport

(Gallemore, 1998). Interestingly, the apically located Na^+/K^+ ATPase pump of the RPE does not contribute to vectorial transport of solute (in parallel with water movement) as it does in other epithelia, but instead is thought to regulate subretinal sodium concentration to support photoreceptor function. The transepithelial transport of chloride plays a major role in driving water movement across RPE, mediated by the Na/K/2Cl- cotransporter on apical membranes and chloride channels present in basolateral membranes (Hughes and Segawa, 1993, Joseph and Miller, 1992).

The high permeability of the RPE to water is enabled by AQP1. Localization of AQP1 to plasma membranes of RPE of human donor eyes and in RPE cells isolated from human donor eyes has been characterized in both fetal and adult (Stamer, et al., 2003). Figure 4 illustrates that decreased AQP1 expression significantly reduces movement of water across fetal human RPE monolayers. It has been demonstrated in rabbits and primates that retinal adhesion can be enhanced by stimulating active transport of fluid across the RPE (Kita and Marmor, 1992, Marmor and Maack, 1982). Thus, the expression of AQP1 by human RPE facilitates water movement that is thought to be critical for sustaining retinal attachment and visual function. Not surprisingly, AQP1 channels are interesting as candidate therapeutic targets for visual disabilities associated with pathological states such as retinal edema.

There appears to be a species difference with respect to aquaporin expression by RPE. While AQP1 mRNA and protein are observed in human RPE, AQP1 protein was not detected in the RPE of rat eyes (Hamann, et al., 1998). The reason for this species difference is unclear, but may be related to differences in eye structure, the presence of

other compensatory pathways for maintaining fluid balance, or the absence of selective pressure for longevity of the visual system in the aging rodent. Consideration of species differences is particularly important given that rats are used as a model organism for studies of transport properties in RPE (Eichhorn, et al., 1996, Maminishkis, et al., 2002). It is likely not a coincidence that all cells that form the blood–ocular barriers (blood–retina and blood–aqueous) and that limit paracellular transport with tight cell–cell junctions, also express aquaporin channels. The RPE, nonpigmented ciliary epithelium and SC endothelium all express at least one aquaporin channel. Such an expression pattern highlights the importance of the efficient water movement across barriers into and out of the eye.

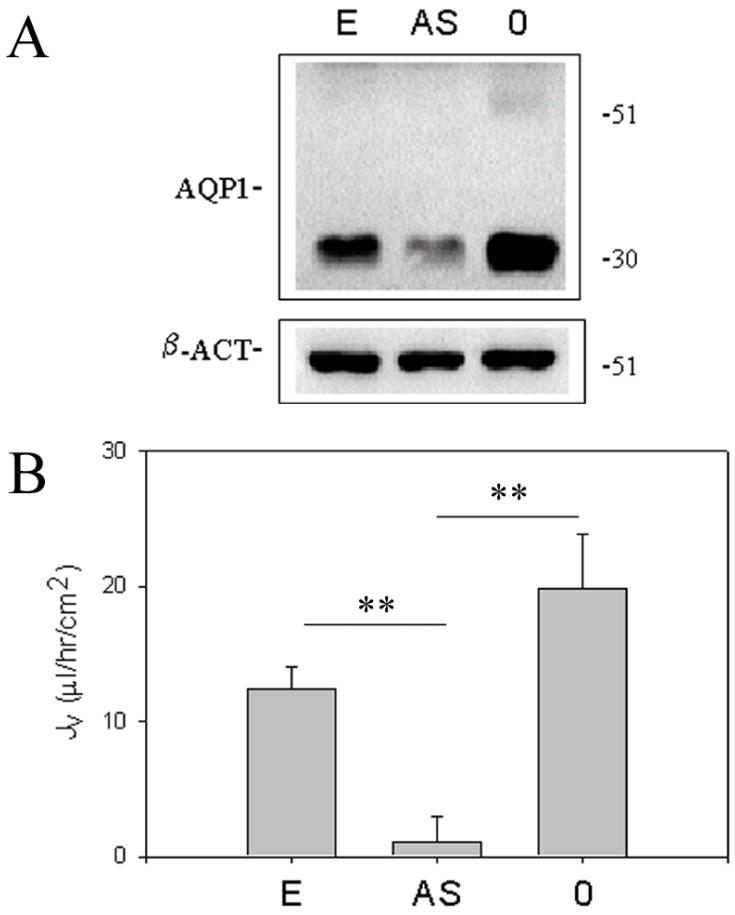

Figure 2.4: Expression and functional analyses of differentiated fetal retinal pigment epithelium monolayer in culture. Panel A shows the expression of native AQP1 in fetal RPE monolayers not infected (0) or after infection with control (empty, E) adenovirus or adenovirus containing antisense AQP1 DNA (AS). Panel B shows amount of water movement across transduced monolayers in response to an osmotic gradient (~150 mOsM). Data are expressed as rate of water movement, J_v (µl/cm^2/hr), Asterisks indicate significant differences between AQP1 expressing monolayers versus control (**p<0.01). *Reprinted from Stamer et al., 2003.*

2.4 Aquaporin-1 Water and Ion Channel Regulation

Aquaporin-1 has been shown to be regulated at various levels including transcriptional and post-translational regulation. At the level of gene regulation, the promoter region of the AQP1 gene contains a hypertonicity response element and conditions of hypertonic stress have been shown to increase AQP1 expression (Umenishi, et al., 2004). Post translational modifications, such as phosphorylation of AQP1 suggest potential regulatory mechanisms of the AQP1 protein (Marinelli, et al., 1997). Increased permeability following phosphorylation of AQP1 indicates that regulation mechanisms are in place to regulate water movement in tissues.

In addition to its constitutive function as a water channel, AQP1 contains a parallel pathway for cations that is regulated in part by the binding of intracellular cGMP (Anthony, et al., 2000, Yool, 2004). Water permeation occurs through individual pores located within single subunits of the aquaporin tetramer, and the central pore at the axis of a four-fold symmetry has been suggested as a candidate ion pore. A conserved internal loop of AQP1 (loop D) has been modeled in molecular dynamic simulations as a flexible gate-like structure that could modify ion permeation at the putative central pore of the tetrameric AQP1 complex (Yu, et al., 2006).

AQP1 channels were shown to carry nonselective monovalent cationic currents after stimulation with PKA (Yool, et al., 1996) and cGMP but not cAMP (Anthony, et al., 2000). When reconstituted in lipid bilayers, AQP1 showed a cGMP-dependent cationic channel function; but only a very small proportion of the total population of water channels incorporated into the bilayer were available to be gated as ion channels

(Saparov, et al., 2001) suggesting that other cellular components were missing in the reconstituted system. Further work has shown that native AQP1 channels in choroid plexus generate a robust cGMP-dependent cationic conductance that is lost after AQP1 knockdown by small interfering RNAs (Boassa, et al., 2006). This cationic conductance activated by atrial natriuretic receptor signaling (and associated cGMP generation) is blocked by Cd^{2+}, and appears to be physiologically relevant in governing fluid secretion. (Boassa, et al., 2006) Recently, similar effects have been shown following stimulation of AQP1 cation conductance with PKC (Zhang, et al., 2007). These data support a physiological role for AQP1 ion channel activity in tissues involved in fluid secretion and absorption. The dual ion and water channel function could in theory allow modification of local osmotic gradients, perhaps enabling adjustments in cell volume and morphology at a microscopic scale, or might serve in signal transduction by causing depolarization of the cGMP-stimulated cells.

In the eye, the importance of AQP1 as a water channel is apparent. An additional role for AQP1 in its mode as a gated cation channel remains to be assessed. Since not all tissues in which AQP1 is expressed would necessarily benefit from Na^{+} entry and the depolarizing effects of the ion channel activity, it is likely that this additional function is under tissue-specific control. The presence of cGMP-sensitive cation channels in tissues of the eye that express AQP1 and are involved in aqueous humor dynamics is an intriguing observation, leading to the speculation that some component of the cation currents could be due to the activity of cGMP-gated AQP1 cation pores. A possible role

for the dual water and ion channel function of AQP1 in the fine control of fluid secretion in ciliary epithelium and RPE is an interesting hypothesis that needs to be tested.

2.5 Aquaporin and Ion Channel Interactions

There is mounting evidence that aquaporins are incorporated into scaffolds at the plasma membrane with other proteins, suggesting that efficient fluid movement across tissues depends not on individual water channels but on complex associations with signaling and transport proteins (Cowan, et al., 2000). In many ocular tissues, chloride secretion provides a key component of the driving force for water movement; however, a parallel pathway for cation flow is required for electroneutral bulk flow. The coexpression of aquaporin water channels and the cystic fibrosis transmembrane conductance regulator (CFTR) channels for chloride enables effective salt and water transport in many types of tissues of the eye, such as corneal epithelia and endothelia, ciliary epithelium, and retinal pigmented epithelium (Levin and Verkman, 2006).

In the ciliary epithelium, sodium enters the pigmented layer from the stromal side along with chloride and transits through gap junctions to the NPE cells for secretion with the aqueous humor primarily through Na^+/K^+ ATPase pumps while chloride exits through various channels (Civan, 2003, Vessey and Kelly, 2004). A possible role for CFTR in chloride movement through the ciliary epithelium is supported by the presence of cAMP-activated chloride currents that result in movement of chloride between the pigmented and nonpigmented epithelium; however, there are conflicting results as to the presence of CFTR in the ciliary epithelium (Chu and Candia, 1985, Do, et al., 2004, Ni, et al., 2006).

Fluid transport across the NPE cells relies on Na^+/K^+ ATPase pump activity and AQP1, as determined by sensitivity to block by mercuric chloride and by antisense knockdown (Patil, et al., 2001).

In the RPE, chloride is the primary driving force for water transport, moving through basal chloride channels including CFTR (Blaug, et al., 2003, Hu, et al., 1996, Miller and Edelman, 1990). Consistent with this idea, humans with cystic fibrosis or mice with mutations in CFTR exhibit decreased chloride transport across the RPE (Gallemore, 1998, Wu, et al., 2006). Less well known are the means by which sodium, the likely counterion to chloride, is moved across the epithelium. Thus, while apical sodium entry is facilitated by the Na/K/2Cl-cotransporter, the basolateral membrane transport mechanism is unknown. The function of AQP1 as an ion channel on either membrane face might augment sodium flux down its electrochemical gradient. An intriguing possibility in both the NPE and RPE is that the cGMP-activated cation flux through AQP1 may modulate net water transport (Fig. 2.5). Because of differences in cellular distribution of Na^+/K^+ ATPase pumps between the NPE and RPE, and the difference in location of the blood supply relative to transport direction across these barriers, it is conceivable that activated AQP1 ionic currents working by the same mechanism would have opposite effects in these two epithelia (i.e., in response to cGMP signaling, slowing the net secretion in the NPE and enhancing net absorption in RPE).

Signaling pathways involving cAMP and cGMP are known to influence salt and water transport in the ciliary epithelium and RPE. Interestingly, while the AQP1 ion conductance is activated by increased cGMP (Anthony, et al., 2000, Boassa and Yool,

2003) it has been suggested to be antagonized by intracellular cAMP (Yool, 2004). Water channel activity of AQP1 is increased by PKA, suggesting that a cAMP-responsive redistribution of AQP1 occurs by phosphorylation of AQP1 (Han and Patil, 2000). It is conceivable that independent regulation of the water and ion channel activity of AQP1 by intracellular signaling cascades would offer intricacy in the control of fluid transport. At present, there is no direct evidence for or against a role for AQP1 ion channels in inflow or outflow pathways, but there are lines of evidence indicating the presence of cGMP-sensitive ionic conductances (Carre, et al., 1996). For example, nitric oxide (NO) and cGMP cause a modest depolarization of the ciliary epithelial transmembrane potential (Fleischhauer, et al., 2001) activate cation conductances in rabbit ciliary epithelium (Carre, et al., 1996), and inhibit Na^+/K^+ ATPase via protein kinase G (PKG) but not PKA (Shahidullah and Delamere, 2006). Each of these instances is consistent with the known ability of NO donors to reduce aqueous humor secretion (Korenfeld and Becker, 1989, Shahidullah, et al., 2005).

Cyclic nucleotide-gated cation channels in the RPE have not been reported; however, chloride and potassium channels in basolateral membranes have been shown to be regulated by intracellular cAMP (Hughes and Segawa, 1993, Joseph and Miller, 1992). Studies have also shown that changes in cGMP levels increase with atrial natriuretic peptide treatment and induce changes in fluid and chloride transport across RPE (Mikami, et al., 1995). Further investigation is necessary to evaluate the mechanisms by which cGMP modulates fluid transport. The relationship between cGMP

and AQP1 provides a potential way to control AQP1 ion channel function and fluid transport across ciliary and retinal epithelia.

In ocular epithelia, the role of cationic currents mediated by AQP1 channels in aqueous humor movement is an interesting possibility that remains to be tested (Anthony, et al., 2000, Yu, et al., 2006). In choroid plexus, the inhibition of Na^+/K^+ ATPase activity and the activation of AQP1 ion channels in response to cGMP stimulation lead to a decrease in net cerebral spinal fluid production; the inhibitory effect is reversed by application of an AQP1 ion channel blocker or by knockdown of AQP1 expression (Boassa, et al., 2006). These data prompt the hypothesis that the braking role of AQP1 ion channel activity on fluid export, in parallel with regulation of the Na^+/K^+ ATPase (Fig. 2.5) might be a conserved theme in the eye and brain ventricle. In ciliary epithelium, AQP1 ion channel activation would be expected to decrease the outflow of water across the membrane, decreasing aqueous humor production. In the RPE, the comparable mechanism of AQP1 ion channel activation will have an opposite effect, serving a complementary role in enhancing net fluid transfer into the RPE for subsequent removal into the blood.

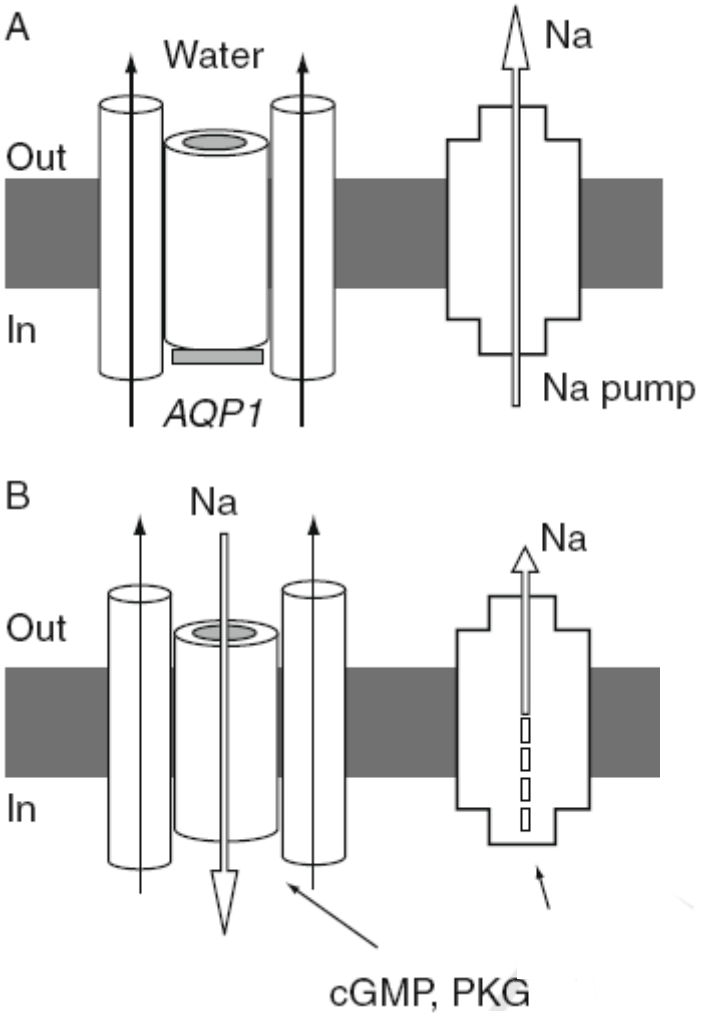

FIGURE 2.5: Hypothetical mechanism for controlling transmembrane salt and water flux by cGMP signaling.
(A) Na active transport out of the cell by the Na^+/K^+ ATPase and water flux through AQP1 water pores in the unstimulated state. (B) Downregulated Na^+/K^+ATPase activity (Ellis et al., 2000) and activation of the AQP1 ionic conductance after cGMP stimulation, resulting in a decrease in net secretion (an increase in net absorption) of fluid via possible local accumulation of Na^+ at the inner membrane.

2.6 Future Directions

While the dependency of aqueous humor secretion on aquaporin expression is clear in aquaporin null mice, such an in vivo model is not ideal for evaluating aquaporin expression in the conventional drainage tract or in the RPE. Thus, experiments are needed that test the role of AQP1 in the physiology and pathophysiology of these two tissues using model systems that more closely resemble the human case. For studying conventional drainage, the human anterior segment perfusion system or live nonhuman primates are commonly used. For studying retinal attachment, a live nonhuman primate model is likely best, unless a lower mammal that expresses AQP1 in the RPE and shows retina edema is identified. The species differences in these two outflow pathways for intraocular fluid is interesting, requires further study, and might offer new insights from comparative physiology into the diversity of strategies that allow management of intraocular pressure and maintenance of ocular homeostasis.

The current information suggests aquaporins in general have the potential to be regulated through multiple mechanisms for a variety of purposes. It is likely that aquaporins, and AQP1 specifically, contributes in previously undefined ways to ocular physiology. The following studies were aimed at characterizing the role of aquaporin-1 in trabecular meshwork and retinal pigment epithelium cultures. Our first study provides new information for the role of AQP1 in trabecular meshwork as a contributing factor to homeostasis during times of mechanical stress. Interestingly, our study of RPE fluid transport suggests that fluid movement previously shown to be mediated by AQP1 in an

RPE culture system is induced via atrial natriuretic peptide, an indirect activator of AQP1 cation currents.

CHAPTER 3

ROLE OF AQUAPORIN-1 IN TRABECULAR MESHWORK CELL HOMEOSTASIS DURING MECHANICAL STRAIN[2]

3.1 Introduction

Glaucoma is the second leading cause of blindness and affects approximately 3 million people in the United States alone (Quigley, 1996). Primary open angle glaucoma is the most common form and is often characterized by an increase in intraocular pressure (IOP). Despite visibly unobstructed conventional outflow tissues, an increase in resistance through the conventional outflow pathway is likely responsible for IOP elevation (Grant, 1963).

The conventional outflow pathway consists of the trabecular meshwork (TM) and Schlemm's Canal (SC), and is part of a dynamic environment subject to multiple forms of environmental stress as well as mechanical strain. Sources of daily mechanical strain in the conventional outflow pathway include intraocular pressure, fluid flow, and contractile activity of surrounding tissues, such as the ciliary muscle. Intraocular pressure and fluid flow are influenced by intraocular processes such as aqueous humor production and drainage (Kaufman, 1984), as well as extraocular processes such as heart beat, eye movement, and blinking (Coleman and Trokel, 1969). The TM is subject to added

[2] Portions of this chapter are currently in press for publication in Baetz, NW, Hoffman, EA, Yool, AJ, Stamer, WD. 2009. Role of aquaporin-1 in trabecular meshwork cell homeostasis during mechanical strain. Exp. Eye Res.

sources of strain as mechanical forces stretch it from Schwalbe's line to the Scleral spur, and inward towards the SC lumen (Johnstone and Grant, 1973). As a result, the TM stretches not only in accordance with changing pressure gradients and fluid movement, but also in conjunction with ciliary muscle contraction (Wiederholt, et al., 2000).

Previous data shows that the TM responds to mechanical stress through a variety of mechanisms. Studies using whole eyes, anterior segments, and isolated TM cultures have demonstrated a range of response mechanisms to mechanical strain. Experiments in eyes of rhesus monkeys and humans revealed reversible structural changes in TM tissues following increases in pressure (Johnstone and Grant, 1973). Perfusion studies using anterior segments of human and porcine eyes reported an increase in outflow resistance (Brubaker, 1975) and a decrease in outflow facility following applied cyclic pressure, suggesting pressure oscillations can induce responses in tissue (Ramos and Stamer, 2008). Investigators have also looked at alterations in cell morphology and actin reorganization following applied mechanical strain to cultured human TM cells (Brubaker, 1975), (Epstein and Rohen, 1991, Mitton, et al., 1997, Tumminia, et al., 1998). Moreover, changes in gene expression have been observed for multiple proteins including myocilin, interleukin factor-6, and matrix metalloproteinases, following applied mechanical strain in TM cultures (Borras, et al., 2002, Bradley, et al., 2003, Bradley, et al., 2001, Liton, et al., 2005, Tamm, et al., 1999, Vittal, et al., 2005). Regulators of transport mechanisms, commonly associated with cell homeostasis are also influenced by mechanical strain in the TM and other tissues (Gasull, et al., 2003). Interestingly, AQP4 has been shown to facilitate increased water flux between muscle tissue and the blood

during increased physical activity, demonstrating a novel role for aquaporin channels during times of mechanical strain (Frigeri, et al., 2004, Frigeri, et al., 2001).

In the conventional outflow pathway, aquaporin-1 (AQP1) has been shown to be expressed in both TM tissue and isolated cultures, though the role of AQP1 in the TM is currently unknown (Hamann, et al., 1998, Stamer, et al., 2008, Stamer, et al., 1995, Stamer, et al., 1994). Since fluid movement through the TM is primarily paracellular, the presence of AQP1 may fulfill a need other than transcellular water movement, particularly in the uveal meshwork where intertrabecular spaces are large. In fact, recent studies have demonstrated that AQP1 does not appear important for bulk fluid movement through the conventional outflow pathway (Stamer, et al., 2008). It is currently unclear as to why robust expression of a water channel protein in the TM would be necessary if fluid movement does not require AQP1 expression. Based upon the unique biomechanical environment of the conventional pathway and recent reports of the role of aquaporins during times of mechanical strain, we hypothesize that AQP1 functions to support TM cell homeostasis during times of mechanical strain.

To test our hypothesis we administered a static and cyclic mechanical stretch to cultured human TM cells and evaluated changes in AQP1 expression and TM cell viability. Our results demonstrate that mechanical strain results in a significant increase in AQP1 expression. To further evaluate the role of AQP1 during mechanical strain we used adenovirus encoding AQP1 to restore AQP1expression in TM cell cultures to levels closer to those observed in TM tissue. We found that increased expression of AQP1 during static mechanical strain reduced measures of cell damage. Our data suggest that

AQP1 in human trabecular meshwork serves a protective role by facilitating improved cell viability during conditions of mechanical strain.

3.2 Materials and Methods

3.2.1 Human Trabecular Meshwork Cells

Five human TM cell strains were isolated and characterized by our laboratory as previously described, (Stamer, et al., 1995) and used for experiments (Table 3.1). Figure 1 shows representative expression of AQP1 seen in TM cultures over time and with passaging. These data confirm that TM cells require multiple days at confluence for maximum expression of AQP1 and that cells cultured up to 14 days retain AQP1 expression and that TM cells used at passage 2 to 4 maintain levels of AQP1 expression. For experiments in the present study, cells were used at passage 2-4 after at least 14 days at confluence. For every experimental condition, we used at least 2 different TM cell lines in at least 3 independent experiments. Listed below are "X" marks designated the cell line used for each type of experiment.

Table 3.1: Cell Strains and Stretch Treatments used in TM study

TM strain	***Donor age***	***Passages***	***Time course***	***10% static***	***20% static***	***8% cyclic***	***13% cyclic***
TM26	15yrs			X	X		
TM86	3mon					X	X
TM88	25yrs	X	X	X		X	X
TM90	4mon	X	X	X	X	X	X
TM92	44yrs	X	X			X	X

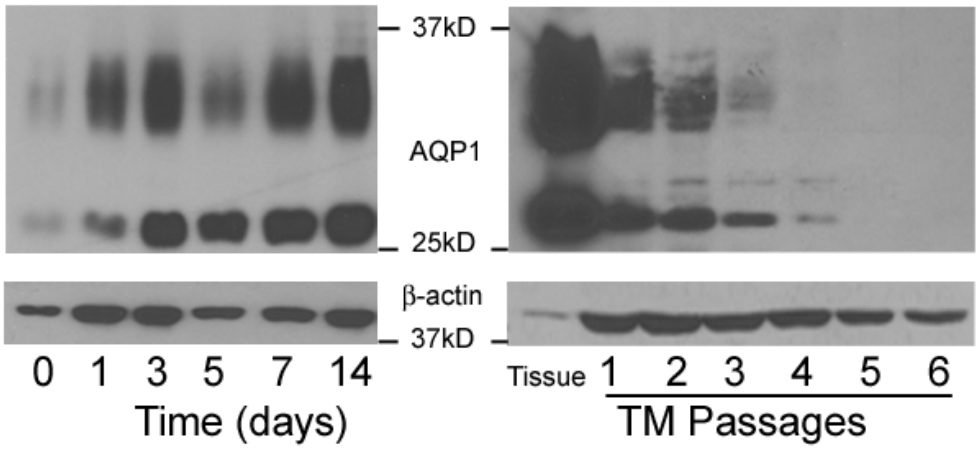

Figure 3.1: Aquaporin-1 expression by trabecular meshwork cell monolayers over time and with passaging. Left panel shows changes in AQP1 expression over time (passage 2) and with passaging (passages 1-6, right panel) compared to AQP1 expression in TM tissue. Displayed are representative western blots for a single cell line (TM90) of three lines total that were tested (table 1).

3.2.2 Stretch Assays

TM cells on Bioflex membrane supports in 6-well format (Product # BF-3001U Flexcell Corporation, Hillsborough, NC) were maintained in 10% fetal bovine serum (FBS, Gemini Bioproducts, Sacramento, CA) low glucose Dulbeco's modified Eagle Media (DMEM, Invitrogen, Carlsbad, CA) supplemented with penicillin (100 units/mL), streptomycin (100mg/mL) and glutamine (0.29mg/mL) for one week after plating at confluence. Cells were then switched to media containing 1% FBS low glucose media for 1 week prior to experiments. Cells were given 2 mL of fresh 0.1% FBS, sodium pyruvate-free media 24 hours prior to and then again during stretch applications. Media was collected before and after stretch to evaluate lactate dehydrogenase (LDH) release. The 6-well plate was placed on a Flexcell stretch frame, housed in an incubator at 37°C

and 5% CO_2. Using Flexcell software, mechanical strains capable of stretching the flexible membranes by 10 and 20% static stretch (stretch and hold), or 9 and 13% Cyclic (oscillatory) Stretch (1 Hz,), were applied by vacuum pump for periods of 8 and 24 hours. The limitations of the equipment allowed only for 9 and 13 % stretch during cyclic stretching at frequency of 1 Hz. Cell lysate samples were collected in 100μL Laemmli buffer at 0, 8, and 24 hours and then boiled for 10 minutes.

3.2.3 Western Blot

Proteins collected from TM cell lysates in Laemmli buffer were loaded on 10 % SDS-PAGE gels run at 0.3 milliamps for 1 hour. The proteins in gel slabs were transferred to nitrocellulose at 100mV for 90 minutes. Following transfer of the proteins, the nitrocellulose was incubated in Tris/HCl-buffered saline (100mM Tris, 137mM NaCl, 2.7mM KCl , pH 7.4) with 2% Tween-20 (TBS-T) and 5% nonfat dry milk (blocking buffer) for 1 hour. The nitrocellulose blots were incubated for 16 hours at 4°C in blocking buffer containing anti-AQP1 IgGs (300 ng/mL). Antibodies were prepared from rabbit serum using affinity purification on Glutathione-S-transferase columns (Stamer, et al., 1995, Stamer, et al., 1996). Blots were rinsed 4 x 15minutes. in TBST, and goat anti-rabbit IgGs conjugated to horseradish peroxidase (Santa Cruz, CA) were incubated with blots in blocking buffer at a dilution of 1:5000 for 1 hour. The solutions containing secondary antibodies were removed and blots were rinsed 4 x 15min. with TBS-T. The blots were then incubated with HyGlo (Denville Scientific, Metuchen, NJ) or Amersham (Buckinghamshire, UK) chemiluminescence western blotting detection solutions.

Following incubation with chemiluminescence reagents, the protein antibody complexes were visualized by timed exposures to X-ray film (Genesee, CA). Following detection of AQP1, the blot was re-probed with solution containing β-actin IgGs (Sigma, St. Louis, MO) raised in mouse and peroxidase-conjugated secondary antibody raised in goat against mouse IgG (Santa Cruz, CA). Signals in the linear range of the film were digitized, and densitometry was performed using Labworks 4 software (UVP, Inc., CA).

3.2.4 Cell Viability Assays

Cell damage and/or death, as determined by release of lactate dehydrogenase (LDH) into conditioned media, was used as an indication of stress levels imposed on TM cells during mechanical stretch. Conditioned media collected from each well was used in analysis of LDH content prior to and following periods of mechanical stretch. Briefly, media were collected, centrifuged at 10,000g for 1 hr, and a 250μL sample was tested for LDH activity. Samples were analyzed using a Roche Cytotoxicity Detection Kit LDH (cat. no. 11644793001) Indianapolis. Media samples of 250μL were mixed with 250μL of the dye/catalyst solution. The dye/catalyst solution consists of catalyst (diaphorase and NAD^+) and dye (Iodotetrazolium chloride and sodium lactate) mixed in a 1:46 ratio and incubated for 30 minutes in the dark. Reduction of NAD^+ to $NADH/H^+$ and subsequent reaction with catalyst allows for the conversion of a yellow tetrazolium salt to a red formazan salt. Each sample was loaded in triplicate into a 96 well plate at 150μL per well. Samples were analyzed using a 490nm filter of Emax precision microplate reader from Molecular Devices (Sunnyvale, CA). Cell death was also assessed by analyzing

conditioned media for the presence of a nuclear enzyme, histone deacetylase. Conditioned media were subjected to centrifugation at 10,000 x g at 4^{o}C for 1 hr. and pellets were solubilized in 100 μL of Laemmli buffer. Histone deacetylase content was analyzed using SDS-PAGE and western blot with commercially available antibodies from Sigma (St. Louis, MO) and Santa Cruz (Santa Cruz, CA) as described above.

3.2.5 Heterologous Aquaporin-1 Expression

Human TM cell monolayers cultured to confluence for 2 weeks were exposed to 1mL of media containing adenovirus (MOI = 1) encoding AQP1 or green fluorescent protein (GFP) 48 hours prior to the start of the stretch for a period of 3 hrs with rocking every ½ hour (Stamer, et al., 2001). Viral media was removed and cells were given 2mL of fresh media containing 1% FBS overnight. After 16 hours, the media was removed and 0.1% FBS sodium pyruvate-free media was added for 24 hours. Media was collected as a "pre-stretch" sample for LDH analysis. Each 6-well plate had two wells that were treated with media alone (uninfected control), two wells infected with GFP adenovirus (virus control), and two wells infected with AQP1 adenovirus. MOI for each virus was obtained by screening virus transduction efficiency in COS cells, having no endogenous AQP1, prior to infection of TM cells. After collection of initial pre-stretch media samples, cells were subjected to 20% static stretch for 24 hours, as previously described. Cell lysates and media samples were collected and analyzed for AQP1 expression and LDH release.

3.2.6 Statistical analysis

Values for aquaporin-1 expression acquired through densitometry were normalized to β-actin expression from experimental and control samples and were analyzed by a two tailed, paired student's t-test. Values for LDH release following stretch were normalized to prestretch control values and analyzed by a two-tailed, paired students t-test. Differences were considered significant at $p < 0.05$.

3.3 Results

3.3.1 Cyclic Stretch Assay

Our first study was designed to evaluate the effect of a cyclic mechanical stretch on AQP1 expression by cultured human TM monolayers. After subjecting TM monolayers to cyclic stretch of either 9% or 13% at a frequency of 1 Hz for periods of 8 and 24 hours, cell lysates were collected and analyzed for AQP1 expression. Application of 1 Hz cyclic stretch reduced the magnitude of stretch achievable on the Flexcell instrument from 10% to 9% and from 20% to 13%. Figure 2A shows representative western blots of AQP1 expression prior to (0) and following either 8 or 24 hours of cyclic stretch. We analyzed the non-glycosylated AQP1 band (28kD) and used β-actin expression as a reference for normalization purposes. Analyses that included both the non-glycosylated and glycosylated AQP1 bands (35kD smear) yielded similar results (data not shown). Figure 2B shows summary data (mean +/- SD) indicating that we observed no significant change in expression at either 9% or 13% cyclic stretch for 8 or 24 hours compared to non-stretched controls.

3.3.2 Static Stretch Assay

We also tested whether a static mechanical stretch of cultured human TM monolayers affected AQP1 expression. Cell lysates from TM monolayers subjected to 10% or 20% static stretch for periods of 8 and 24 hours were analyzed for AQP1 protein expression. Figure 3A shows a representative western blot of AQP1 expression from TM cell lysates prior to (0) and following 8 and 24 hours of static stretch. Normalized data

(mean+/- SD) of AQP1 expression for 10% and 20% static stretch is shown in the summary graph in figure 3B. A significant 2-fold increase was observed in AQP1 expression following the 10% stretch for 8 and 24 hours ($p<0.05$). Similarly, we observed at least a 3.5-fold increase in AQP1 expression following the 20% stretch for 8 and 24 hours ($p<0.05$).

3.3.3 Lactate Dehydrogenase Assay

The presence of lactate dehydrogenase (LDH) in media was used as an indirect measure of stress experienced by TM cells during mechanical stretch. Media collected before and after stretch were compared to negative control (no stretch) and positive control (1% Triton lysis) samples. Significant amounts of LDH were detected in conditioned media following 24 hours 13% cyclic ($p = 0.044$) and 20% static ($p = 0.002$) stretch. With both of these two types of stretch we observed a ~2 fold increase in LDH compared to control. Viability was also tested by probing western blots with primary antibodies against histone deacetylase (HDA), a nuclear enzyme. Interestingly, we observed no difference in the appearance of HDA between any of the control and experimental groups (data not shown). Taken together, results indicate that the levels of mechanical strain applied to cells in the present study induced some degree of cell damage, but not global cell death.

3.3.4 Heterologous Expression of Aquaporin-1 and Trabecular Meshwork Viability

To test the importance of AQP1 expression in TM cell homeostasis under conditions of stretch, we used adenovirus encoding recombinant AQP1 to bring total AQP1 levels closer to physiological levels (Figure 3.5a). Because we observed that static stretch had the most consistent effect on TM cell viability, we expressed recombinant AQP1 in TM cells and administered a 20% static stretch for 24 hours. Media was collected prior to and following 24 hours of static stretch and analyzed for LDH activity. Figure 5b shows that LDH from AQP1 infected cells was 2-fold lower in comparison to GFP control cells ($p = 0.03$). Interestingly, when analyzed together with uninfected cells, these data demonstrate an inverse linear relationship between AQP1 expression and LDH in conditioned media in response to stretch ($r^2 = 0.7780$).

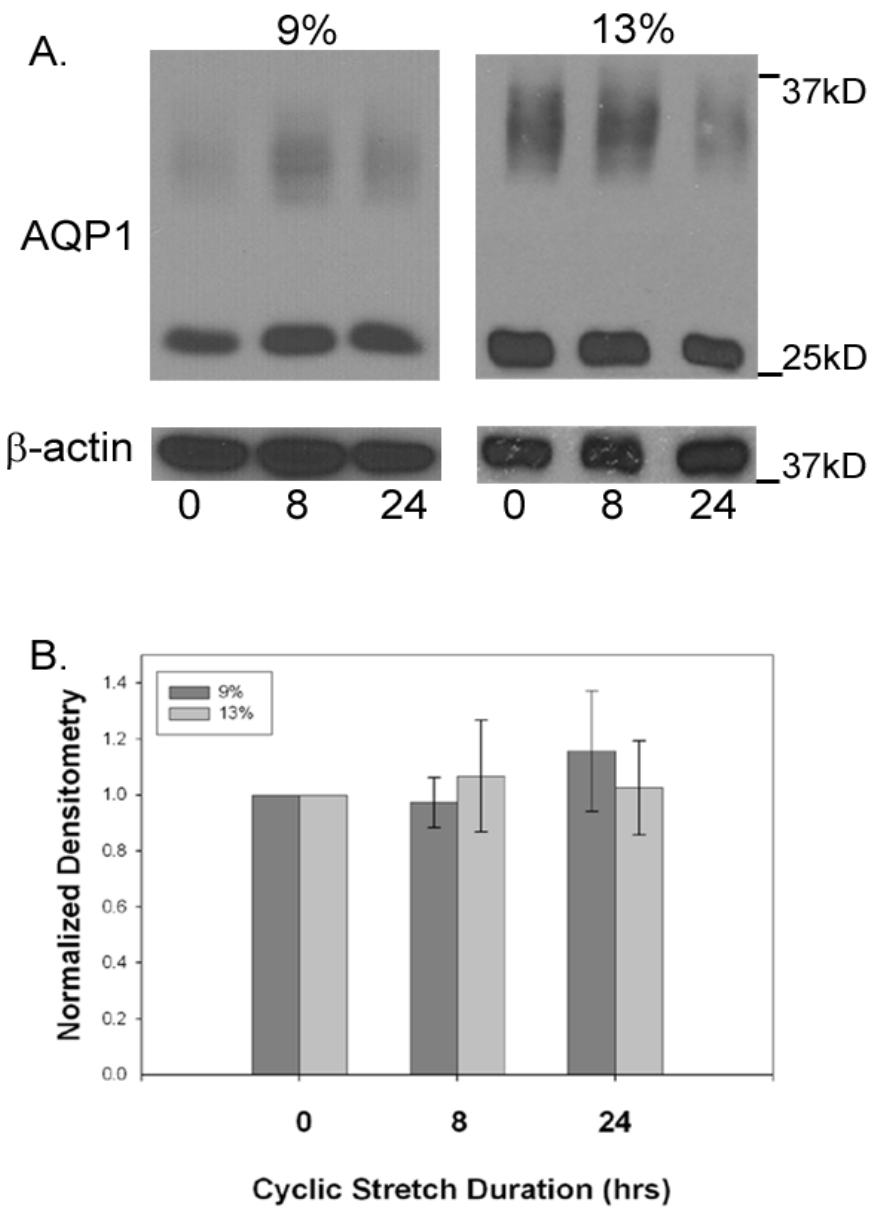

Figure 3.2: Effect of cyclic stretch and aquaporin-1 expression by trabecular meshwork cell monolayers. TM cells plated at confluence and maintained for two weeks on Bioflex membranes were subjected to cyclic stretch (9% or 13%) at 1 Hz for 8 or 24 hours. Cell lysates at 0, 8, and 24 hours were collected and analyzed by SDS-PAGE/western blotting using affinity purified antibodies specific for AQP1 (panels A). Panel B shows a histogram of AQP1 expression levels over time of exposure to stretch that were normalized to β-actin prior to stretching. Data represent combined densitometry data (mean ± SD) from 4 independent experiments.

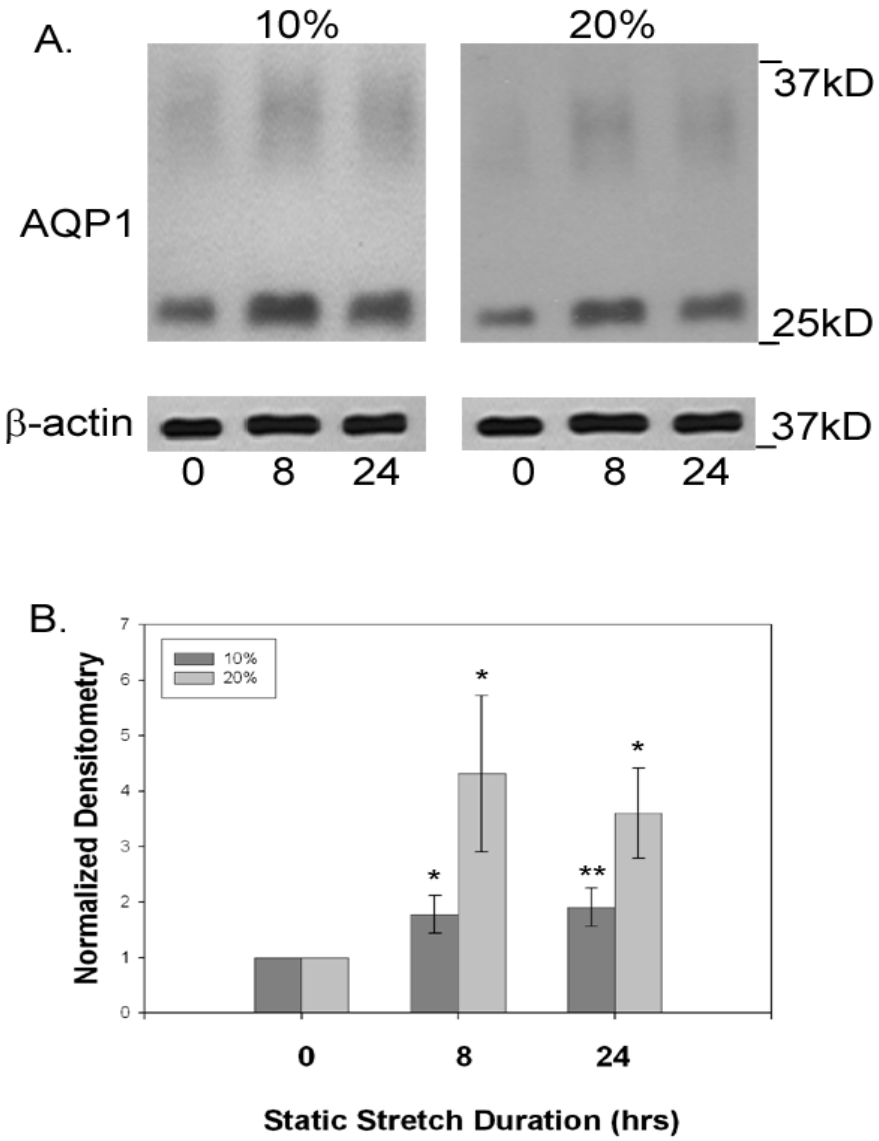

Figure 3.3: Effect of static stretch on aquaporin 1 expression by trabecular meshwork cell monolayers. TM cells plated at confluence and maintained for two weeks on Bioflex membranes were subjected to static stretch (10 or 20%) for 8 or 24 hours. Cell lysates at 0, 8, and 24 hours were collected and analyzed by SDSPAGE/western blotting using affinity purified antibodies specific for AQP1 (panel A). Panel B shows a histogram of AQP1 expression levels over time of exposure to stretch that were normalized to β-actin prior to stretching. Data represent combined densitometry data (mean ± SD) from 4 independent experiments (*$p<0.05$).

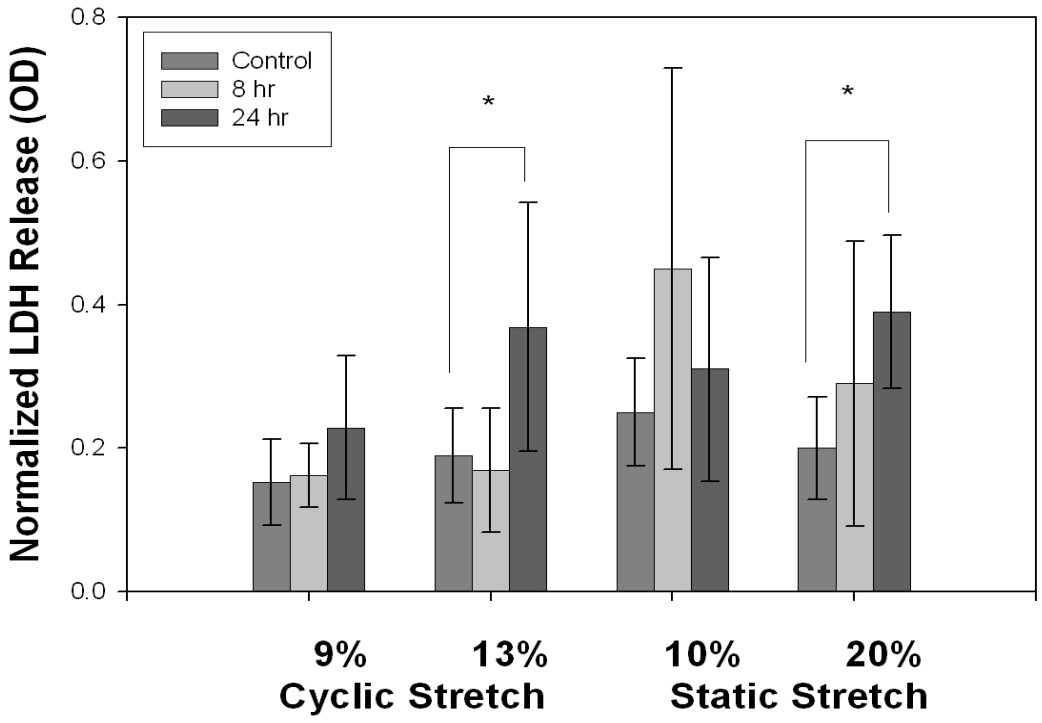

Figure 3.4: Viability of trabecular meshwork cells exposed to cyclic or static stretch. TM cells plated at confluence and maintained for two weeks on Bioflex membranes were then subjected to no stretch (control), static stretch (10 or 20%) or cyclic stretch (9 or 13% at 1Hz). Conditioned media was collected after 8 or 24 hours of stretch and analyzed for lactate dehydrogenase (LDH) content. Shown are combined data (mean ± SD) from 4 independent experiments that were normalized to optical density (OD) values for total lysis controls (*$p<0.05$).

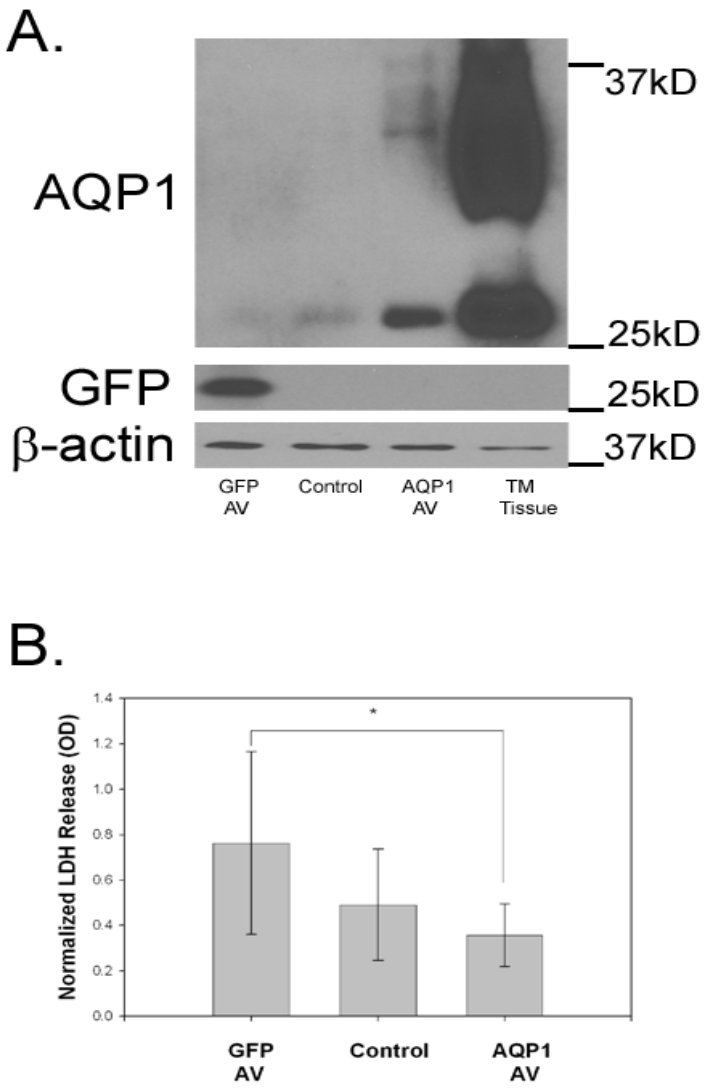

Figure 3.5: Viability of rabecular meshwork cells exposed to 20% static stretch following expression of recombinant aquaporin-1. TM cells plated at confluence and maintained for two weeks on Bioflex membranes were either not transduced (control) or were transduced with GFP or AQP1 adenovirus and subjected to 20% static stretch for 24 hours. Conditioned media was collected after 24 hours of stretch and analyzed for lactate dehydrogenase (LDH) content. Cell lysates were collected and analyzed by SDS-PAGE/western blot using affinity purified antibodies for AQP1, GFP and β-actin (Panel A). Shown in panel B is a histogram of combined data (mean $\pm$ SD) from 5 independent experiments that were normalized to optical density (OD) values for total lysis controls (*$p<0.05$).

3.4 Discussion

The purpose of our study was to examine the role of AQP1 in TM cells during mechanical strain. TM cells responded to static mechanical stretch by increasing AQP1 expression. Interestingly, cyclic mechanical stretch did not significantly impact AQP1 expression. Consistent with a role for AQP1 in cell homeostasis, the most significant impact on cell viability was found during static mechanical stretch. We observed that LDH release from TM cells was inversely proportional with the level of AQP1 expression. Taken together, our data suggest that AQP1 serves a protective role in human TM cells during static mechanical stretch.

Our studies were designed to approximate physiological levels of static stretch in vivo by applying a stimulus of 10 and 20% to cultured human TM cells. With static stretch we attempted to model two physiological stressors experienced by the TM, ciliary muscle contraction and/or elevated IOP. Earlier studies have shown that IOP elevations in rhesus monkeys result in as much as a 50% stretch of TM and SC cells in the JCT region (Ethier, 2002, Grierson and Lee, 1977). Qualitative studies of sectioned human conventional outflow tissue however, demonstrate less distention in the TM in comparison to the SC (Grierson and Lee, 1974), in part due to the lack of giant vacuoles which allow for larger increases in surface area (Grierson and Lee, 1975). We note that 10% stretch is commonly used by other groups and estimated to be within the physiological range experienced by TM cells (Chow, et al., 2007, Mitton, et al., 1997, Tamm, et al., 1999, Tumminia, et al., 1998, WuDunn, 2001). Consistent with this idea,

we observed elevations in LDH release from cells exposed to higher stretch magnitudes for longer times, but did not see global cell death as measured by histone deacetylase. These data further suggest that though the stretch magnitudes used in the current study are estimated to approximate the mechanical strain in vivo, the TM is likely subjected to much greater levels of stress in vivo. It is important to note that the TM in vivo is also subject to a variety of other stresses associated with phagocytosis of cellular debris and exposure to oxidative biproducts in the anterior chamber which may induce cell damage or death that might not be achievable with the stretch system alone (Liton and Gonzalez, 2008).

The cyclic mechanical stretch in the current studies was intended to simulate changes associated with ocular pulse. Previous studies, particularly in TM cultures have used a 10% cyclic stretch and estimated this level of stretch to be within a physiological range (Chow, et al., 2007, Mitton, et al., 1997, Tamm, et al., 1999, Tumminia, et al., 1998, WuDunn, 2001). To our knowledge, studies have not examined the level of stretch experienced by the TM in response to ocular pulse. However, studies in other tissues such as pulmonary capillaries have suggested that 5% stretching of endothelial cells is within a physiological range (Birukov, et al., 2003, Birukova, et al., 2006). We were not able to detect a change in AQP1 expression at either the 9 or 13% levels, yet we did see an increase in LDH release during 13% cyclic mechanical stretch; indicating that the cells were experiencing physical strain. The reason that TM cells increased AQP1 expression in response to both 10 and 20% static stretch, but not cyclic stretch at either the 9 or 13% levels is unclear.

We structured the present study to test our novel hypothesis that AQP1 accommodates rapid changes in TM cell volume during mechanical strain to maintain TM homeostasis. A precedent for the role of aquaporins in cell volume regulation has already been demonstrated in the TM as well as other tissues (Chan, et al., 2004, Krane, et al., 2001, Liu, et al., 2006, Mitchell, et al., 2002, Stamer, et al., 2001). It would be interesting to determine in future studies the connection between cell volume regulation and cell viability during mechanical strain. Moreover, it would be of interest to determine if in some glaucomas, AQP1 levels are unusually low, making TM cells more vulnerable to mechanical insults such as elevated IOP (Alvarado, et al., 1984). Characterizing the mechanisms responsible for TM maintenance and protection is critical for improving our general understanding of TM physiology as well as providing potential targets for treatment of TM cells that are subject to mechanical as well as other environmental stressors.

CHAPTER 4

THE EFFECT OF ATRIAL NATRIURETIC PEPTIDE ON RETINAL PIGMENT EPITHELIUM FLUID TRANSPORT

4.1 Introduction

The retina is attached in the posterior of the eye by adhesive forces that prevent retinal detachment and the potential loss of vision (Marmor and Maack, 1982, Marmor and Yao, 1994). Adhesion of the retina to the underlying retinal pigment epithelium and choroid is dependent upon a combination of intraocular pressure, extracellular matrix between the retina and retinal pigmented epithelium (RPE) (Yao, et al., 1990), and transport processes across the RPE (Michels, 1990). Active transport of solutes across the RPE into the blood is important for the steady export of fluid from the eye to the blood across the RPE barrier to keep the extracellular fluid space small, facilitating retinal attachment, while allowing the exchange of extracellular medium needed to maintain an appropriate ionic and metabolic environment for the photoreceptor cells (Steinberg, 1979). Accumulation of fluid in the subretinal space as a result of damage to the retina can lead to retinal detachment and ultimately blindness. Disruption of water and ion transport across the RPE inhibit recovery to normal volumes in the subretinal space following retinal detachment (Kang and Luff, 2008). Understanding the regulation of volume in the subretinal space is important for efficient treatment of retinal detachment.

Several classes of channels and transporters are involved in RPE transport. The apical localization of the Na^+/K^+ ATPase has been shown in bovine and human RPE and has been proposed to provide for the accumulation of sodium into the subretinal space to support the background dark current of the photoreceptors (Hu, et al., 1994, Joseph and Miller, 1991, Miller, et al., 1978). Paradoxically, while the sodium is pumped into the subretinal space by the Na^+/K^+ ATPase, the net fluid flow measured in RPE under physiological conditions is in the apical to basal direction. This directional export of fluid has been proposed to facilitate retinal attachment , and is mediated predominantly by anion and cation influx through the apically located Na/K/Cl cotransporter (Adorante and Miller, 1990, Hu, et al., 1996). The cotransporter serves as the primary route for apical chloride uptake; basal chloride channels provide a means for chloride to move across the RPE, creating the transepithelial salt transport needed as the major driving force for water transport (Bialek and Miller, 1994). Potassium is thought to serve as a counter ion for chloride in baseline RPE fluid absorption process, yet this seems more plausible at the basal membrane than for net transport (Hughes and Takahira, 1996, Segawa and Hughes, 1994, Takahira and Hughes, 1997). Though epithelial sodium channels (ENaC) are presumably a large part of this net solute and fluid movement, the involvement of these channels is not well understood (Wimmers, et al., 2007).

Regulatory pathways for ion and water transport in the RPE have been demonstrated by identification of receptors as well as characterization of signaling pathways. Identification of adrenergic receptors in human and bovine RPE support the potential for receptor mediated signaling to regulate RPE transport (Frambach, et al.,

1990, Frambach, et al., 1988, Joseph and Miller, 1992). Agonists such as ATP and epinephrine stimulate a variety of processes in RPE including potassium and chloride transport, fluid transport, and calcium signaling indicating previously identified adrenergic receptors are important for RPE physiology (Edelman and Miller, 1991, Hughes, et al., 1987, Hughes, et al., 1988, Hughes, et al., 1984, Hughes and Segawa, 1993, Hughes and Takahira, 1998, Maminishkis, et al., 2002, Peterson, et al., 1997). Epinephrine activates adenylyl cyclases increasing intracellular cAMP levels. Studies of cAMP effects on RPE differ between species and when cAMP treatment showed a reduction in apical to basal RPE fluid transport in bovine (Miller, et al., 1982), whereas studies of cGMP in rabbit eyes indicate an increase apical to basal fluid transport (Marmor and Negi, 1986). In line with this evidence is a study demonstrating atrial natriuretic peptide (ANP) treatment of RPE rabbit cultures increases chloride uptake in cultured RPE (Mikami, et al., 1995). Furthermore, this effect was reduced by an inhibitor of Protein Kinase G, suggesting atrial natriuretic peptide signaling likely increases intracellular cGMP which activates PKG to increase chloride transport. These data provide a precedent for ANP signaling in the RPE and a contribution to chloride movement, the primary driving force for water transport in the RPE.

Atrial natriuretic peptide regulation of fluid movement in the human body has been shown in a number of tissues (Potter, et al., 2006, Tremblay, et al., 2002), most notably the heart and kidney, but also in the brain (Johanson, et al., 2006). Secretion of cerebrospinal fluid across the choroid plexus cultures was reduced upon treatment of with atrial natriuretic peptide (Steardo and Nathanson, 1987). Briefly, atrial natriuretic peptide

is a 28 amino acid peptide primarily produced in the cardiac myocytes, though evidence suggests there are local sources of the peptide in the eye (Wolfensberger, et al., 1994). Atrial natriuretic peptide binds a membrane bound guanylyl cyclase and increases intracellular levels of cGMP. Aquaporin-1 studies in primary culture of choroid plexus cells indicated that part of the reduction in CSF secretion in response to atrial natriuretic peptide is due to a cation current through AQP1, activated by cGMP (Boassa, et al., 2006). Interestingly, the choroid plexus and the RPE share functions as a transporting epithelium, the unique apical localization of the Na^+/K^+ ATPase, and expression of AQP1. These data raise the question as to whether AQP1 ion channel function may play a role in fluid absorption across the RPE.

The current study was designed to address whether atrial natriuretic peptide influences apical to basal fluid movement in RPE; if the signaling induced by atrial natriuretic peptide is mediated by a cGMP/PKG dependent pathway; and if the AQP1 ion current facilitates ion and ultimately water flux across RPE monolayers. My results confirm that atrial natriuretic peptide increases apical to basal fluid movement in cultured RPE, and that this signaling is mediated by a cGMP dependent pathway which is not blocked by PKG inhibitors, in contrast to the data previously shown for PKG in the rabbit RPE (Mikami, et al., 1995). Combined, the new information supports a role for atrial natriuretic peptide induced fluid transport, but indirectly implicates targets of cGMP other than PKG.

4.2 Methods

4.2.1 Retinal Pigment Epithelium Culture Model

Retinal pigment epithelium from a human donor was prepared as previously described (Davis, et al., 1995, Hu and Bok, 2001). As documented in these prior studies, these monolayers maintain polarity, resistance, and pigment just as RPE in vivo do. Briefly, newly proliferated cells from primary tissue in a low calcium media were collected every 3-4 days and frozen in 10% DMSO/10% FBS DMEM over a period of approximately 4 weeks. Cells were thawed to give ~300,000 cells per 12mm Millicell HA filter (Millipore, Billerica, MA) coated with approximately 1-2ug of mouse laminin. Cells were cultured for a period of 8-10 weeks. RPE monolayers used in my studies had an average net resistance of 324 Ohms*cm^2 and the resistances ranged from approximately 75 to 900 Ohms*cm^2. Figure 4.1 shows representative images of RPE pigmentation over 8-10 weeks

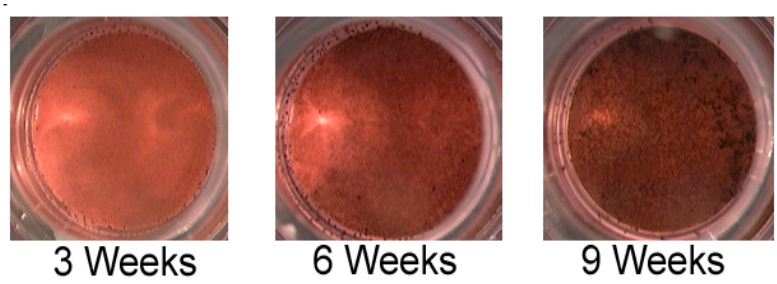

Figure 4.1: Retinal pigment epithelium photos of pigmentation over time.
Representative photos of RPE pigmentation over time. (photos from RPE cell line 4967)

4.2.2 Transepithelial Resistance (TER) Measurement:

Prior to treatment, transepithelial resistances (TER) of RPE monolayers were recorded using World Precision Instruments Voltohmeter (Sarasota, FL). Control resistances were recorded on filters with no cells and subtracted from total resistance of monolayers. Figure 4.2 shows representative TER recordings from 2 cell lines. Groups refer to RPE monolayers plated at the same time on individual filters. Variation between groups is representative of the variation in TER seen between groups of filters.

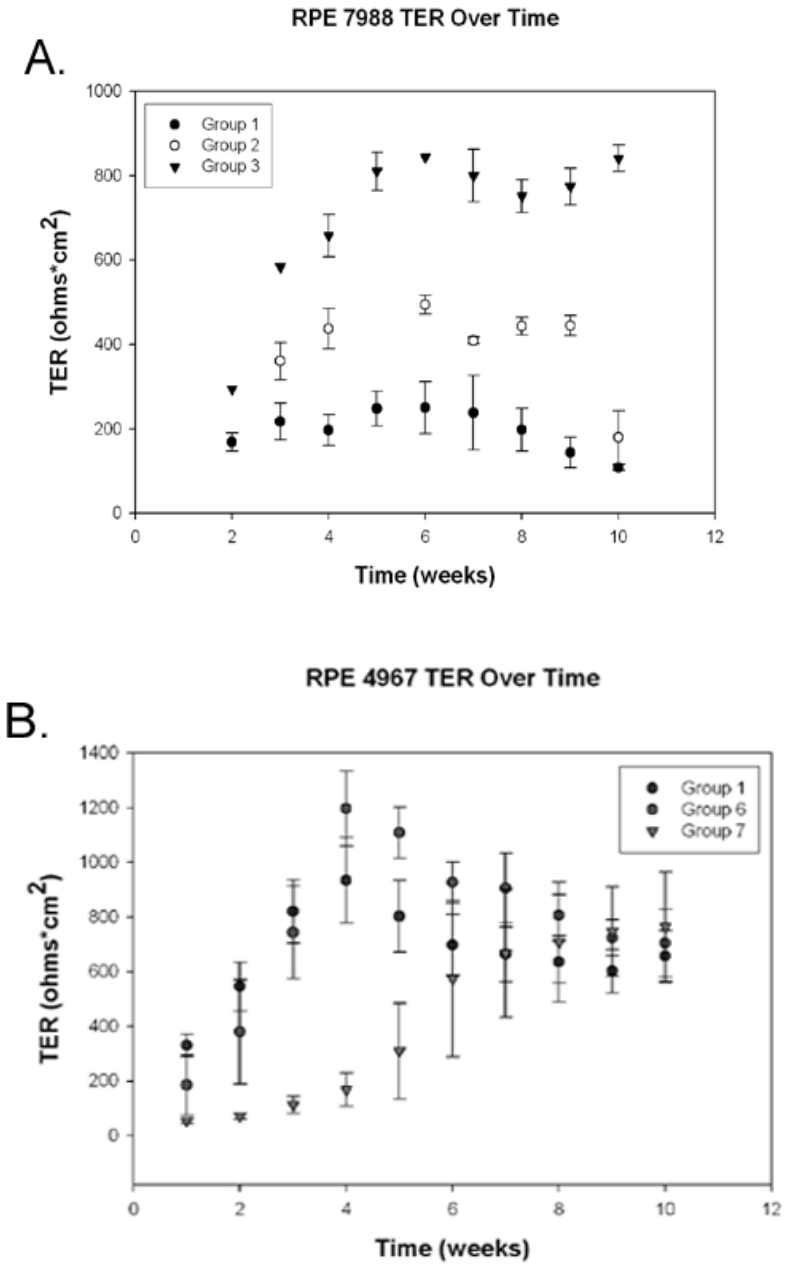

Figure 4.2: Retinal pigment epithelium transepithelial resistance measurements over time. Panels A and B. RPE cell line 7988 and 4967; Representative TER recordings of three groups of monolayers from 2 cell lines over 8-10 weeks.

4.2.3 Atrial Natriuretic Peptide and Anantin Fluid Transport Assay

Three or more RPE monolayers were incubated with 150μL of RPE media (Hu and Bok, 2001) added to the apical chamber and 1mL added to the basal chamber to measure baseline fluid transport for 2 hours at 37°C. Media from the apical chamber was then collected and weighed to calculate the amount of fluid displaced from the apical chamber in 1 hour. RPE monolayers were incubated in 500μL media for 1 hour at 37°C before further treatment. Following measurement of baseline fluid transport across RPE monolayers, the same monolayers were incubated a second time with 150μL of media containing 4.5μM ANP (Sigma, St. Louis CAT # A1663). Media from the apical chamber was collected and weighed to calculate the amount of fluid displaced from the apical chamber in 1 hour. RPE monolayers were rinsed three times with media and allowed to equilibrate for 1 hour. Inhibition of ANP induced fluid transport was performed using a combination treatment of ANP (4.5μM) and Anantin (45μM) (Sigma, St. Louis, CAT# A4316) in 150μL of media for 2 hours at 37. Media from the apical chamber was collected and weighed to calculate the amount of fluid displaced from the apical chamber in 1 hour.

4.2.4 8-Bromo-cyclic Guanosine Monophosphate and H-8 Fluid Transport Assay

Fluid transport with treatment of 8-Bromo-cyclic Guanosine Monophosphate (8-Br-cGMP) was conducted same as described above for atrial natriuretic peptide assay.

Following measurement of baseline fluid transport RPE monolayers were then incubated a second time with 150μL of media containing 2mM 8-Br-cGMP (Sigma, St Louis CAT # B1381). Media from the apical chamber was collected and weighed to calculate the amount of fluid displaced from the apical chamber in 1 hour. RPE monolayers were rinsed three times with media and allowed to equilibrate overnight before further treatment. Inhibition of cGMP induced fluid transport was determined by first repeating baseline fluid transport studies to ensure a return to baseline fluid movement across the monolayers with a 1 hour equilibration period, followed by a treatment with H-8 (N-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride PKG inhibitor, 2mM) (Calbiochem, San Diego, CAT #371958) for 30 minutes in 150μL media at 37°C. The media was then removed and the same RPE monolayers were treated with a combination of 8-Br-cGMP (2mM) and H-8 (2mM) for 2 hours at 37°C. Media from the apical chamber was collected and weighed to calculate the amount of fluid displaced from the apical chamber in 1 hour.

4.2.5 8-Br-cGMP Dose Dependent Fluid Transport Assay

Dose dependent measurements of 8-Br-cGMP induced fluid transport across RPE monolayers were made using of 8-Br-cGMP (50μM, 500μM, 2mM, 5mM, 10mM). Baseline fluid transport was measured as previously described for the atrial natriuretic peptide assay. Each dose of 8-Br-cGMP was tested three times on three replicate RPE monolayers. Media from the apical chamber was collected and weighed to calculate the amount of fluid displaced from the apical chamber in 1 hour.

4.2.6 8-Br-cGMP induced Fluid Transport vs. Hydrostatic Gradient

Fluid transport against a hydrostatic pressure was tested by increasing the volume of media in the basal chamber from 1 to 2mL and repeating the measurements as described above. These experiments were conducted three times on at least three RPE monolayers each time with 2mM dose of 8-Br-cGMP for 2hours at 37°C. Media from the apical chamber was collected and weighed to calculate the amount of fluid displaced from the apical chamber in 1 hour.

4.2.7 Protein Kinase G Assays

Protein Kinase G activity was assayed using Cyclex Cyclic GMP dependent protein kinase (cGK) Assay Kit (CAT# CY-1161, MBL International, Woburn, MA). Briefly, a purified catalytic domain of PKG was incubated with 125μM ATP in the presence or absence of H8 as well as a peptide substrate phosphorylated by activated PKG. PKG and ATP were removed after 30 minutes and the substrate was rinsed, incubated with anti-phospho antibody, provided with the PKG assay kit, for 1 hour, rinsed and incubated for 5 minutes with substrate reagent. Stop Solution of was added after five minutes and colorimetric change was measured with a Molecular Devices 96 well plate reader at 450nM.

4.2.8 Statistical Analysis

Values for fluid transport between treatments and controls were analyzed using paired students t-test. Differences were considered significant at $p < 0.05$.

4.3 Results

4.3.1 Effect of Atrial Natriuretic Peptide on Retinal Pigment Epithelium Fluid Transport

My first study was designed to determine if ANP affected RPE fluid transport. Net fluid displacement from the apical side was determined by weight after a 2 hour incubation period at 37°C in the absence or presence of ANP. Specificity was confirmed with Anantin, an antagonist of the ANP-receptor, which blocked the stimulatory effect of ANP. Figure 4.3 shows that compared to baseline (-0.58 $\pm$ 2.65 $\mu L/hr*cm^2$), net fluid movement increased with ANP treatement of RPE monolayers (12.60 $\pm$ 2.11 $\mu L/hr*cm^2$) ($p = 0.02$, $n = 3$). Co-incubation of ANP and Anantin on RPE monolayers resulted in a decrease in this effect. Anantin (45μM) inhibited the ANP stimulated fluid transport (5.19 $\mu L/hr*cm^2$) (n=2). Using the same methods, I conducted a fluid transport assay using 100 μM dose of adenosine triphosphate (ATP) to demonstrate that ATP has similar effects in the system presently used as in other RPE model systems (Maminishkis, et al., 2006). In fact figure 4.4 shows that fluid transport was significantly increased in ATP treated (10.70 $\pm$ 1.92 $\mu L/hr*cm^2$) RPE monolayers in comparison to control (4.17 $\pm$ 4.07 $\mu L/hr*cm^2$) ($p < 0.01$, $n = 3$); r*esults = mean* $\pm$ *SD.*

4.3.2 Effect of 8-Bromo-cyclic Guansine Monophosphate on Retinal Pigment Epithelium Fluid Transport

To further characterize the signaling responsible for the effect of ANP on RPE fluid movement, we evaluated fluid transport following treatment of RPE monolayers with 2mM 8-Br-cGMP. RPE monolayers were treated using the same protocol, with baseline fluid movement measured first followed by treatment periods. Figure 4.5 shows that in comparison to baseline fluid flow (2.87 ± 2.73 $\mu L/hr*cm^2$), the treatment of RPE monolayers with 2mM 8-Br-cGMP significantly increased apical to basal fluid movement (21.35 ± 1.59 $\mu L/hr*cm^2$), ($p<0.0005$, $n=3$). Application of H-8 did not significantly inhibit 8-Br-cGMP induced fluid movement (19.69 ± 6.77 $\mu L/hr*cm^2$), ($p = 0.56$, $n=3$). This data indicates that Protein Kinase G (PKG) activation is not necessary for net fluid transport in an RPE culture model. To evaluate the efficacy of the H-8 inhibitor we measured PKG phosphorylation of a peptide substrate using purified catalytic domain of PKG in the presence of ATP or ATP with H-8. Figure 4.6 shows that PKG phosphorylation of the substrate (1.74 ± 0.20) as measured by optical density was significantly reduced ($n=3$ $p \leq 0.05$) in the presence of H8 (0.23 ± 0.13). This data indicates that the H8 inhibitor does effectively reduce kinase activity.

4.3.3 Dose dependent Effect of 8-Bromo-cyclic Guanosine Monophosphate on Retinal Pigment Epithelium Fluid Transport

A dose dependent effect of 8-Br-cGMP on RPE fluid transport was assessed (50μM, 500μM, 2mM, 5mM, 10mM). Fluid transport was measured as previously described with a baseline control for fluid movement prior to each dose. The dose response curve for the effects of 8-Br-cGMP on fluid movement indicates an EC_{50} value of 2.5mM cGMP showing that the dose of 2mM 8-Br-cGMP used in the previous assays was appropriate. Figure 4.7 illustrates the dose response measured in this assay.

4.3.4 Effect of 8-Bromo-cyclic Guanosine Monophosphate against a Hydrostatic Pressure

The possible contribution of hydrostatic pressure to the fluid fluxes was determined by repeating the fluid transport assays with 2mM 8-Br-cGMP but increasing the basal volume to 2mL, predicted to generate a basal to apical hydrostatic pressure of approximately 0.003mmHg. Our results indicate the apical to basal fluid movement in treated (15.18 ± 5.05 $\mu L/hr*cm^2$) monolayers that persisted against the hydrostatic back pressures was significantly different ($n=3$; $p \leq 0.05$) than baseline values (4.54 ± 2.48 $\mu L/hr*cm^2$) and was not significantly ($n=3$; $p = 0.15$) different than those values observed after cGMP treatment with 1mL volume in the basal chamber (17.14 ± 2.48 $\mu L/hr*cm^2$). The data suggest that the fluid movement originally observed in response to treatment with 8-Br-cGMP is not due to hydrostatic pressure.

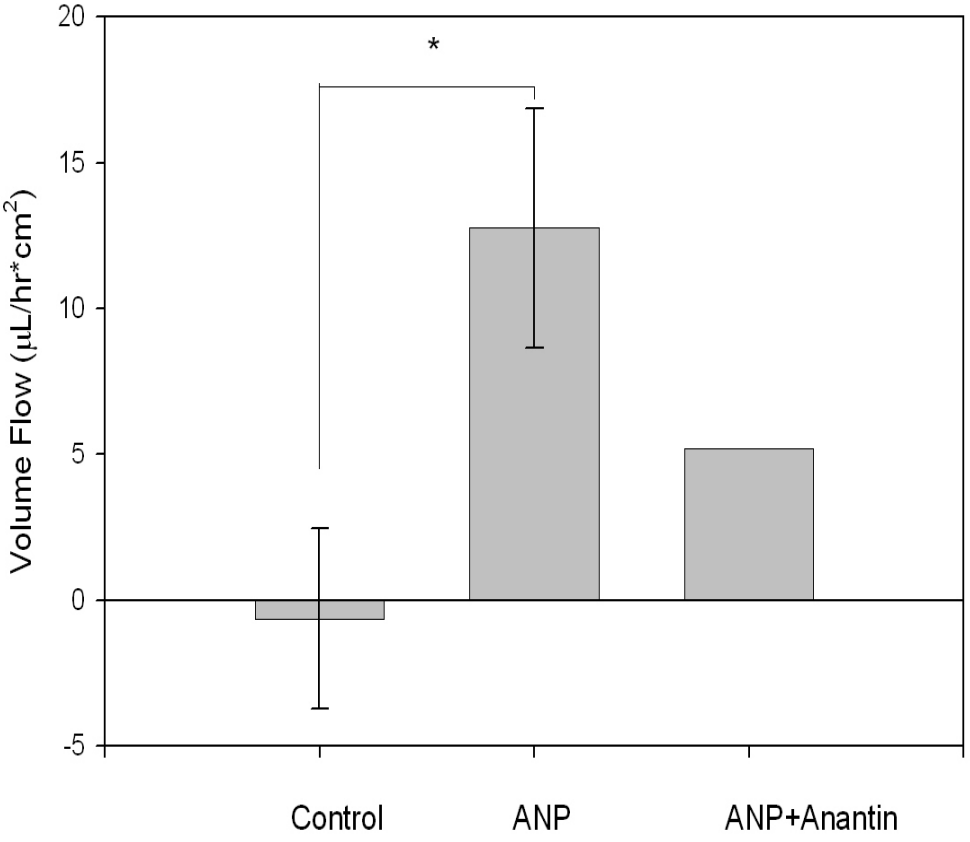

Figure 4.3: The effect of atrial natriuretic peptide on retinal pigment epithelium fluid transport Volume flow in control (-0.58 ± 2.65 µL/hr∗cm^2) vs. ANP treated (12.60 ± 2.11 µL/hr∗cm^2) RPE monolayers was significantly different ($p < 0.019$, $n = 3$); Anantin (45µM) inhibited RPE fluid transport (5.19 µL/hr∗cm^2) (n=2). r*esults = mean± SD*

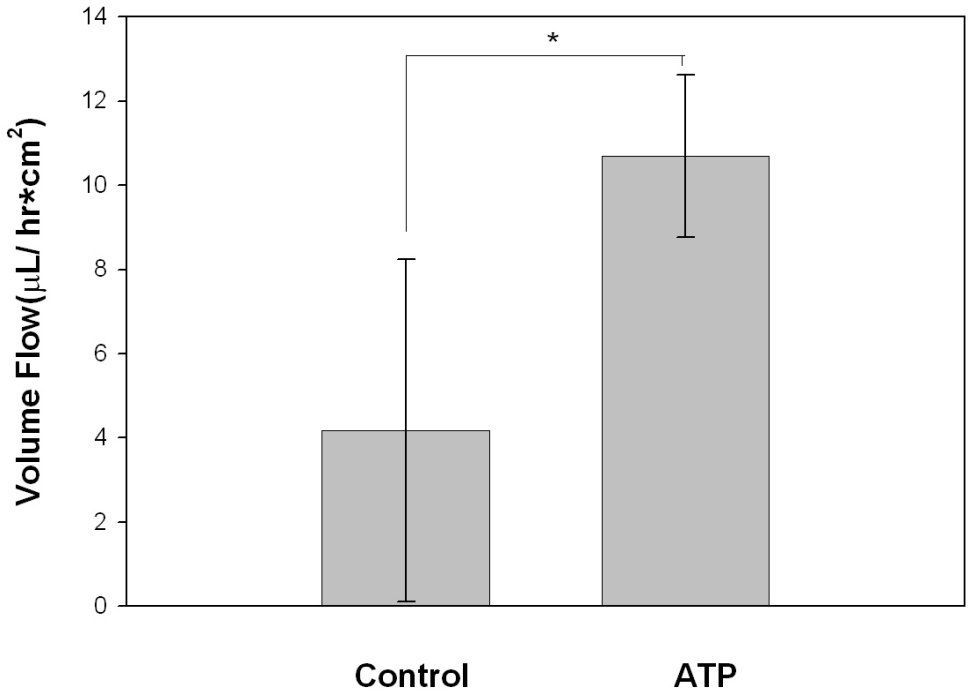

Figure 4.4: The effect of adenosine triphosphate on retinal pigment epithelium fluid transport Volume flow in control (4.17 $\pm$ 4.07 μL/hr$*$cm^2) vs. ATP treated (10.70 $\pm$ 1.92 μL/hr$*$cm^2) RPE monolayers was significantly different ($p < 0.01$, n = 3); r*esults = mean$\pm$ SD*

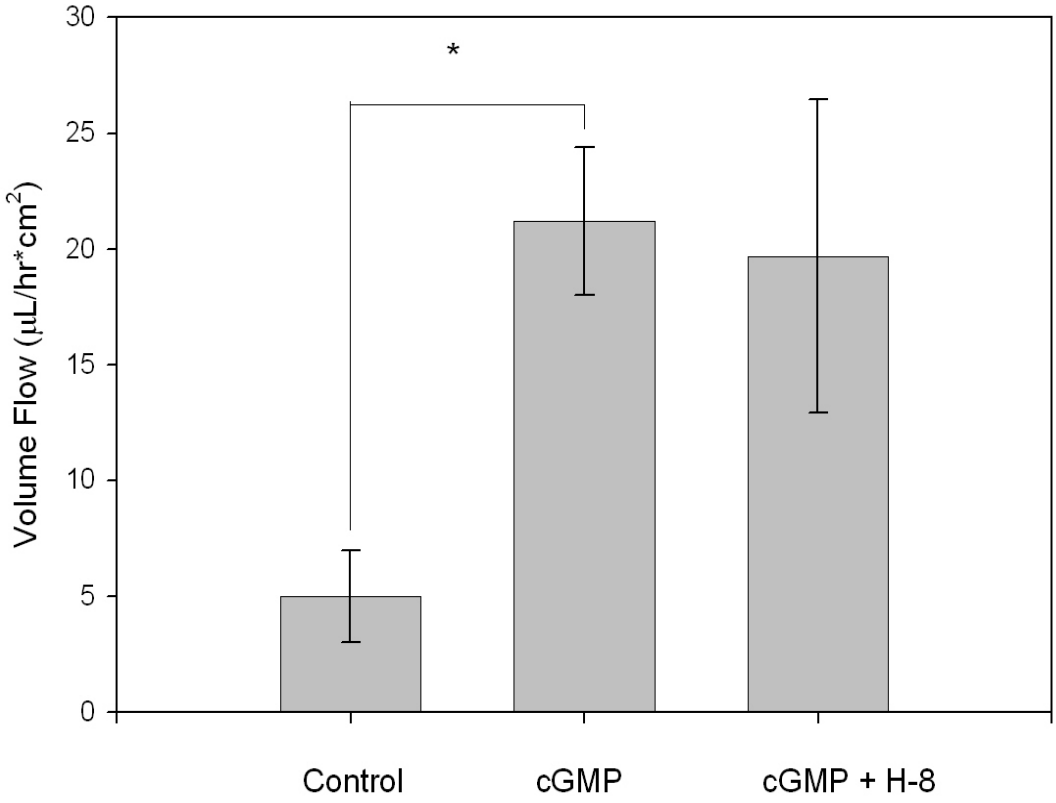

Figure 4.5: The effect of 8-bromo-cyclic guanosine monophosphate on retinal pigment epithelium fluid transport Volume flow in control (2.87 $\pm$ 2.73 µL/hr$*$cm^2) vs. 8-Br-cGMP treated (21.35 $\pm$ 1.59 µL/hr$*$cm^2) RPE monolayers was significantly different ($p < 0.0005$, n=3) *results= mean$\pm$SD* H-8 did not inhibit RPE fluid movement (19.69 $\pm$ 6.77 µL/hr$*$cm^2) ($p = 0.56$, n=3).

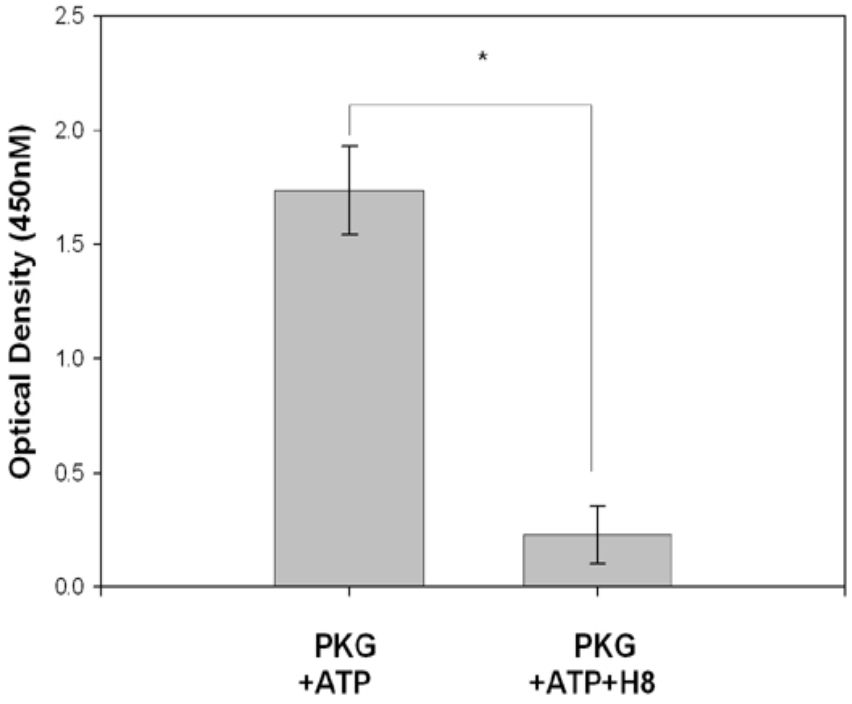

Figure 4.6: The effect of H-8 on protein kinase G activity
H8 significantly (n=3 $p \leq 0.05$) reduces the PKG activity (0.23 ± 0.13) in comparison to control (1.74 ± 0.20) as determined by optical density absorbance reading (450nM) of phosphorylated substrate treated with phospho-antibody and substrate reagent.
r*esults = mean± SD*

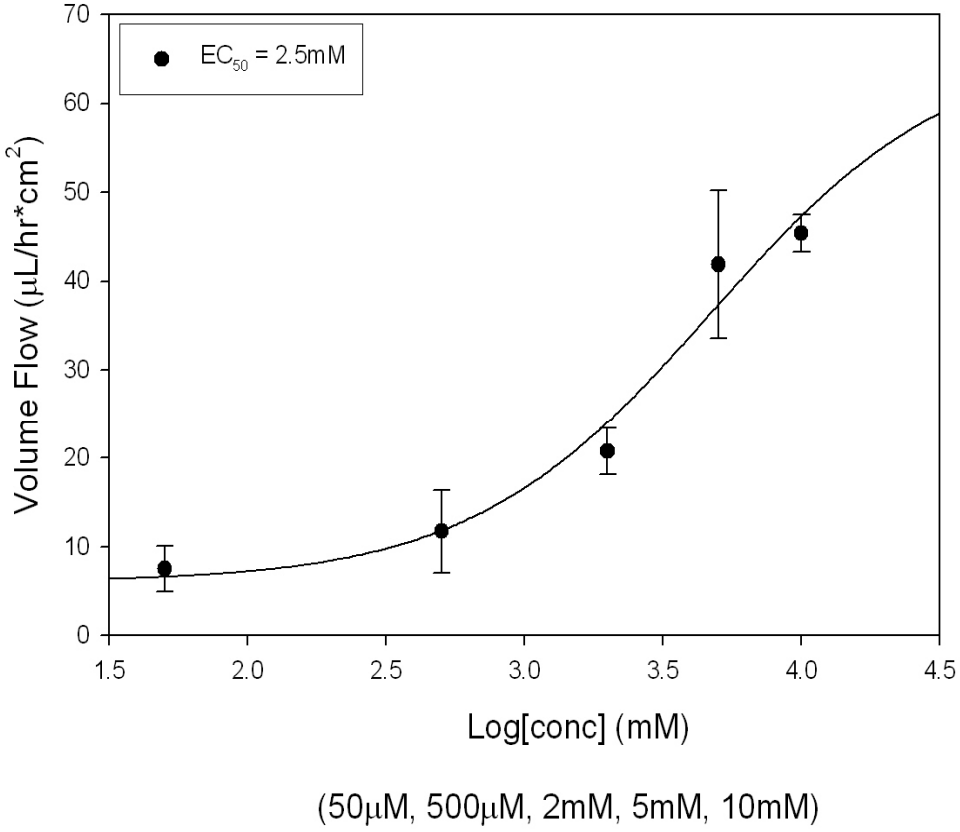

Figure 4.7: Dose repsonse of retinal pigment epithelium monolayers to 8-bromo-cyclic guanosine monophosphate RPE monolayers treated with 5 concentrations of 8-Br-cGMP showed a dose dependent response. The EC_{50} is 3.4, approximately 2.5mM 8-Br-cGMP. r*esults = mean± SD*

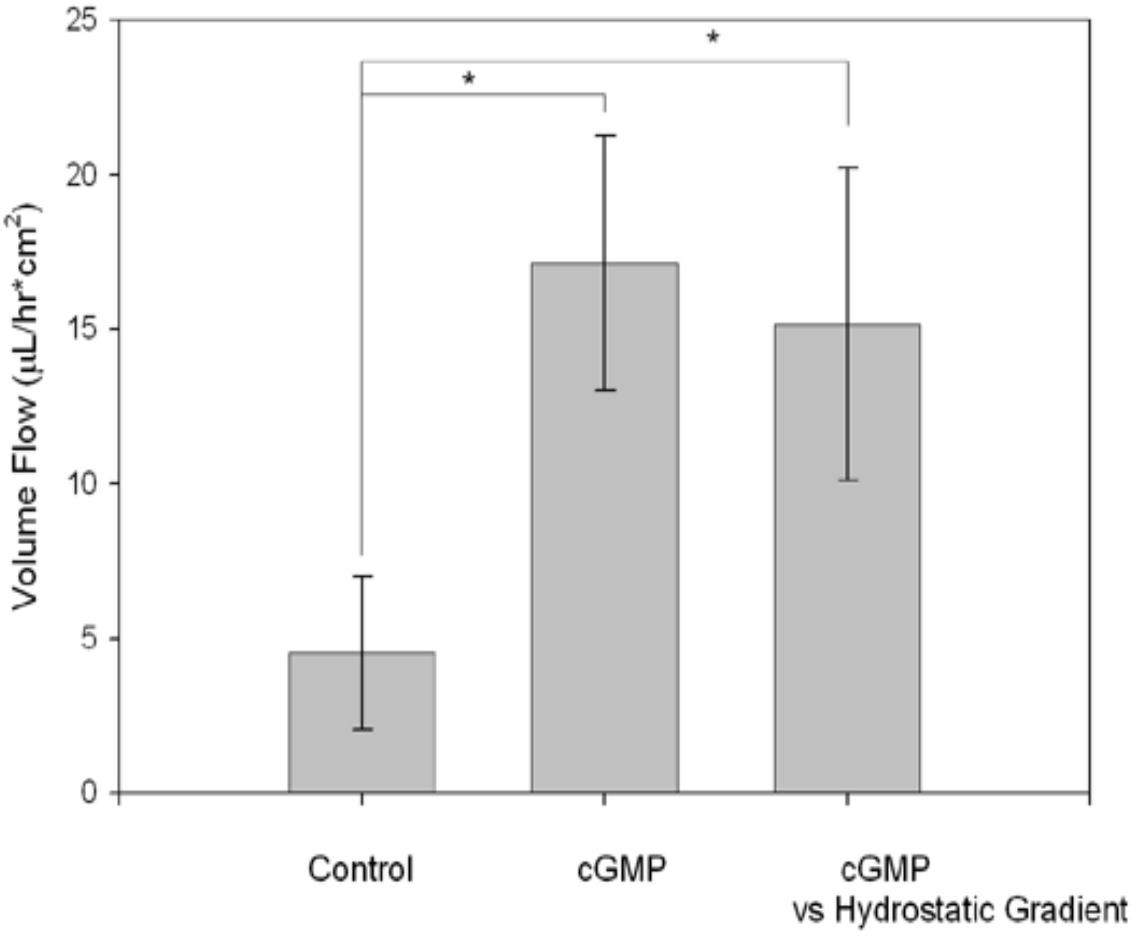

Figure 4.8: Effect of 8-bromo-cyclic guanosine monophosphate on retinal pigment epithelium fluid transport against a hydrostatic pressure. RPE monolayer fluid transport against a hydrostatic pressure ($15.18 \pm 5.05 \mu L * cm^2$) was significantly different ($n=3$; $p \leq 0.05$) than control ($4.54 \pm 2.48 \mu L * cm^2$) but not significantly different then monolayers treated in the absence of a hydrostatic pressure ($17.14 \pm 4.11 \mu L * cm^2$). r*esults = mean ± SD*

4.4 Discussion

The current study confirmed that ANP increased RPE fluid transport through cGMP signaling. Stimulation of RPE monolayers with ANP and 8-Br-cGMP resulted in an increased apical to basal net fluid transport. To assess the signaling pathways responsible for the stimulatory effect in RPE, I used H-8 as an inhibitor of PKG, and observed no significant difference in net fluid transport, suggesting cGMP mediated fluid transport is mediated by a kinase-independent pathway. Furthermore, the data presented support an active process of fluid transport based on the consistent apical to basal fluid movement against a hydrostatic pressure. Of interest are prior studies that demonstrated the regulation of AQP1 cation channel activity was due to a direct action of cGMP, independent of kinase activity (Anthony, et al., 2000). Taken together, the data suggest ANP stimulates RPE fluid transport by a cGMP dependent pathway that does not appear to involve PKG.

Primary cultures of RPE cells on permeable supports and cultured for 8-10 weeks developed increased resistances. The RPE culture system used here (Davis, et al., 1995, Hu and Bok, 2001) is considered to be the best available having been empirically optimized to simulate numerous features of RPE in vivo including hexagonal morphology of the cells, transepithelial resistances (~1000 ohms$*$cm^2) that approach resistances of RPE in vivo (~2000 ohms$*$cm^2), localization of characteristic proteins, such as the Na^+/K^+ATPase (Hu, et al., 1994), and expression of pigmentation with maturity. The investigation of RPE fluid transport against a hydrostatic pressure further supports

the tightness of these monolayers contributing to the similarities with RPE in vivo. Additionally, it is important to note that previous studies in rabbit have reported similar amounts of fluid transport (~16μL/hr∗cm^2) per hour following treatment of rabbit eyes with 1mM 8-Br-cGMP (Marmor and Negi, 1986). The combined past and present studies indicate that the RPE model system employed for the current studies responds similarly to 8-Br-cGMP as other previously established systems used for physiological studies of the RPE.

The present data show a functional response to agonist of ANP receptors in the RPE monolayers. Previous work has shown the natriuretic peptide receptors for ANP and BNP peptides are present in human retina and RPE (Rollin, et al., 2004, Wolfensberger, et al., 1994). Furthermore, studies in RPE cultures have indicated that ANP significantly increases intracellular cGMP levels in comparison to treatment with SNP, an activator of soluble guanylyl cyclase (Diederen, et al., 2008, Diederen, et al., 2007) suggesting that the activation of the membrane bound guanylyl cyclase is the preferred pathway for increasing intracellular levels of cGMP in RPE. Studies demonstrating RPE secretion of ANP and subsequent activation of cGMP synthesis in RPE cells further support the presence and possible relevance of this signaling pathway in the RPE (Diederen, et al., 2008). Collectively, the precedent for natriuretic peptide signaling in the RPE and the new data presented here support a role for ANP induced fluid transport in the RPE.

Physiologically relevant levels of cyclic nucleotides are estimated to be within the micromolar range in vivo following activation of guanylyl or adenylyl cyclases

(Smolenski, et al., 1998), suggesting our doses may be above the physiological range. Interpretation of the relevance of the results obtained with an apparently supra-physiological dose of 8-Br-cGMP (2 mM) requires consideration of two points. First, the micromolar doses are for intracellular actions, whereas the agent applied in my studies is extracellular and thus the effective intracellular dose is unknown. Membrane permeability of analogs of second messengers differs; the 8-Br-cGMP analog is less lipophilic than newer analogs such as Rp-8-Br-PET-cGMP which contain larger hydrophobic side groups that increase membrane permeability (Schwede, et al., 2000). Second, it is important to note that previous studies also used millimolar concentrations of 8-Br-cGMP during perfusion of rabbit eyes indicating these levels are needed in order to deliver enough agonist (Marmor and Negi, 1986). My dose response studies of cGMP in the RPE monolayers indicates that the concentration used is just below the half maximal effective concentration defined empirically. Taken together, the doses of 8-Br-cGMP used in the current study, appear to be below saturating levels for evaluating effects on fluid transport.

The inhibition studies using H-8 for inhibition of PKG did not reduce net fluid transport, supporting the possibility of other targets for cGMP, such as the AQP1 cation channel. Implication of AQP1 in mediating RPE fluid transport has been previously shown (Stamer, et al., 2003). I anticipate that a contribution of the AQP1 cation channel in RPE would result in an increase in net fluid movement in the apical to basal direction. In fact, this is exactly what was observed in the current study. Also, no cyclic nucleotide gated channels aside from the potential AQP1 cation channel have been characterized in

the RPE. Of particular interest are any channels that might contribute to cation flux. The exact mechanism of sodium transport in the RPE is not understood. Epithelial sodium channels are believed to play a role, but the identification of these channels in RPE has not been demonstrated (Wimmers, et al., 2007). A contribution from AQP1 as a cation channel would perhaps contribute to large net flux of sodium and other potential cations, such as potassium. The fact that the observed increase in fluid transport is not reduced using PKG inhibitors would be explained by direct targets for cGMP.

The prevention and treatment of retinal detachment is aimed at reducing retinal edemas following damage to the retina. The new findings here are the first to demonstrate a role in RPE fluid transport for ANP signaling and suggest that targets downstream of cGMP aside from PKG may be important in fluid transport. These data provide a new possibility for regulating RPE fluid transport and increasing fluid movement from the subretinal space into the blood. The studies also provide a potential candidate channel that may contribute to RPE solute movement. All of the results presented here provide new information regarding basic biology of RPE fluid transport and also potential therapeutic implications for pathologies associated with retinal detachment.

CHAPTER 5

SUMMARY, INTERPRETATION, AND FUTURE STUDIES

5.1 Summary

The combined studies provide new evidence regarding the role and regulation of AQP1 mediated ocular fluid movement. The first study showed that AQP1 expression in TM cells is increased with static mechanical stretch and that TM cell damage is reduced as AQP1 expression is restored to near-tissue levels. The second study showed that fluid transport across RPE monolayers, through previously identified AQP1 channels (Stamer, et al., 2003), is increased by ANP and cGMP and that inhibition of PKG does not reduce this effect. Taken together, the data demonstrate that AQP1 fulfills tissue-specific requirements in ocular tissues in response to external mechanical and molecular cues.

In the first study AQP1 expression was increased with 8 and 24 hour periods of static mechanical stretch. The results show that AQP1 expression significantly increased by 2 fold with 10% static stretch and 3.5 fold with 20% static stretch at 8 h ($n=4$, $p<0.05$) and 24 h ($n=6$, $p<0.05$). While histone deacetylase levels were unaffected by treatments, release of LDH from TM cells was the most profound at the 20% static stretch level ($n=4$ $p<0.05$). Significantly, cells were refractory to the 20% static stretch level when AQP1 expression was increased to near tissue levels. Analysis of LDH release with respect to AQP1 expression revealed an inverse linear relationship ($r^2 = 0.7780$). Therefore,

correlative changes in AQP1 expression and TM viability depend upon the magnitude and type of imposed mechanical strain.

In the second study fluid transport across RPE monolayers that express AQP1 is increased with treatments of ANP and cGMP. The results showed that compared to the baseline flow rate (-0.58 $\pm$ 2.65 $\mu L/hr*cm^2$), flow across ANP treated (12.60 $\pm$ 2.11 $\mu L/hr*cm^2$) RPE monolayers increased significantly ($p = 0.02$, $n = 3$). Anantin (45μM) inhibited the ANP stimulated fluid transport (5.19 $uL/hr*cm^2$) (n=2). Compared to baseline volume flow (2.87 $\pm$ 2.73 $\mu L/hr*cm^2$), flow across 8-Br-cGMP treated (21.35 $\pm$ 1.59 $\mu L/hr*cm^2$) RPE monolayers increased significantly ($p<0.0005$, n=3). Application of H-8 did not significantly inhibit 8-Br-cGMP induced fluid movement (19.69 $\pm$ 6.77 $\mu L/hr*cm^2$) ($p = 0.56$, n=3). Thus, ANP increases fluid movement through cGMP dependent signaling, that is not dependent upon PKG.

The collective results of this study demonstrate a tissue specific response for the role and regulation of AQP1 water channels in ocular tissues. The trabecular meshwork, which is routinely subjected to sources of mechanical strain, increases the expression level of AQP1, one result of which is the prevention of cell damage. The current data support an alternative role for AQP1 mediated fluid transport in the TM outside of bulk fluid movement. In contrast, the RPE serves a traditional role as an epithelial barrier tissue where bulk fluid movement is mediated by AQP1. The RPE however, shares an apical localization of the Na^+/K^+ ATPase with only one other epithelium in the human body, the choroid plexus. Interestingly AQP1 cation conductance has been shown to

contribute a solute drag effect in the choroid plexus following treatment with ANP. Net fluid transport in the RPE occurs in the opposite direction, thus a contribution of AQP1 cation conductance would be expected to increase net fluid transport in the apical to basal direction. In accordance with this idea, treatment of RPE cultures with ANP and cGMP increased apical to basal fluid movement providing new information about signaling mechanisms that regulate RPE fluid transport and preliminary support for AQP1 in RPE as a cation channel. The current study provides new information regarding the potential roles for AQP1 in ocular tissues in response to various stimuli and indicate that AQP1 is not limited to constitutive water movement across cells, but rather is involved in regulation of homeostasis though both traditional (water movement) and non-traditional (ion movement) processes.

5.2 Interpretation

The present work demonstrates the potential roles for AQP1 in different ocular tissues. While serving a traditional role of transcellular fluid movement in the RPE, AQP1 mediated fluid movement serves a unique role in the TM, providing a protective effect during periods of mechanical strain. The TM is subject to routine stress from oxidative biproducts in the aqueous humor outflow, phagocytosis of debris, and mechanical stress (Liton and Gonzalez, 2008). The mechanical strain imposed on the TM can result from normal physiological events including ocular pulse, ciliary muscle contraction, and blinking or squinting (Coleman and Trokel, 1969). Furthermore, pathological conditions, such as those associated with increases in intraocular pressure,

can also result in mechanical stress in the outflow pathway. The TM adapts to respond to these changes through stretching and contraction in order to accommodate the changes in surrounding tissues and environment. Previous studies have demonstrated that the TM responds to periods of mechanical strain by changing outflow facility and resistance and even by increasing cell number (Ramos and Stamer, 2008). Interestingly studies have also indicated a volume regulation response mechanism in TM cells that express AQP1 (Mitchell, et al., 2002, Stamer, et al., 2001). The present study suggests that increased levels are important specifically during static mechanical stretch indicating the duration and magnitude of stretch may require a corresponding response from the TM. One possible explanation for the differences observed in the TM study is that a sustained pressure or force on the TM requires increased permeability so that TM cells can absorb some of the volume that may be associated with an increase of intraocular pressure. Based on the new data, I hypothesize that AQP1 channels in the TM facilitate rapid changes in cell volume in response to periods of mechanical strain in order to prevent cell damage. The daily changes in stress load that the TM experiences would require mechanisms sufficient to promote TM tolerance and the ability to adapt to recurring influences that might otherwise compromise the tissue.

The RPE, which is an epithelial barrier, functions to support the retina and balance the composition of fluid and solutes in the subretinal space for proper photoreceptor function and retinal attachment. The environment around the RPE is not subjected to the same dynamic mechanical strain as the TM. The RPE shares more similarity with other epithelial tissues such as the choroid plexus. In fact, the RPE and

choroid plexus are unique in comparison to other epithelium based on localization of transporters and channels. Interestingly, studies in the choroid plexus have shown that AQP1 cation conductance is physiologically relevant in reducing fluid secretion. Because of the similarities between the RPE and choroid plexus, the current study was designed to determine if an AQP1 cation conductance might be important in the RPE. The possibility of an AQP1 cation current in the RPE stimulated by ANP treatment is supported by previous studies where cGMP treatment reduced the size of blebs induced under rabbit retinas suggesting cGMP stimulates an apical to basal fluid movement (Marmor and Negi, 1986). The current investigation provided evidence that ANP increases apical to basal net fluid movement through a cGMP dependent mechanism that does not appear to be dependent upon activation of Protein Kinase G. This line of evidence supports the possibility of another target for intracellular cGMP. There are multiple targets for cGMP including ion channels gated by cyclic nucleotides, phosphodiesterases, and protein kinase G, which subsequently targets calcium pumps and the inositol-triphosphate receptor (IP3R) (Schwede, et al., 2000, Smolenski, et al., 1998). Though cyclic nucleotide gated (CNG) channels would provide the most direct answer for the effect observed in the current study, there is no precedent for this type of channel in the RPE. The CNG channel is characteristic of photoreceptors and currently, AQP1 is the only channel in the RPE known to be directly regulated by cGMP. A feedback mechanism of cGMP dependent activation of phosphodiesterases could potentially decrease levels of cyclic nucleotides that influence RPE fluid transport. Due to many targets of cGMP as well as cAMP, further study in this area would help solidify the

effects of these two second messengers. Additionally, the activity of PKG has been shown not only to influence transporters, such the Na/K/Cl cotransporter and the Na^+/K^+ ATPase in the plasma membrane, but also calcium pumps in the plasma membrane and endoplasmic reticulum which would effectively reduce intracellular levels of calcium. Because calcium signaling is thought to increase chloride transport the major driving force of RPE fluid transport, PKG may also reduce fluid transport. Though multiple mechanisms may be responsible for cGMP induced fluid transport, the present data, provide new information regarding the potential role of AQP1 in RPE fluid transport and open the possibility that AQP1 cation conductance may play a role in contributing to ion flux as well as water flux across the RPE.

5.3 Future Studies

The new information in the current study can be advanced by future studies that characterize the role and regulation of AQP1 in ocular tissues. The study of AQP1 in the TM culture system during mechanical strain would be furthered by completing the following experiments. First it would be important to determine if the role of AQP1 as a contributor to homeostasis could be perturbed with addition of a blocker for AQP1. We would expect that blocking the AQP1 water channel during a static stretch for example would prevent water flux in and out of these cells, leading to an increase in cell damage and possibly cell death despite a parallel increase in AQP1 expression. Second, investigation of how cell volume changes during periods of mechanical strain in the presence and absence of a specific blocker for AQP1 would support a role for the water

channel in volume regulation during stress. Third evaluation of the expression of AQP1 by in situ hybridization in control versus glaucomatous eyes would provide insight as to how the water channel is regulated in vivo under pathological conditions. Given the potential activity of AQP1 in response to ANP in epithelial tissues, it may also be interesting to determine if ANP has an effect in TM cells, particularly during mechanical strain. Studies have shown ANP has an effect in aqueous humor secretion, but no effect has been established in TM or the outflow pathway. Overall it would be important to investigate how a rapid recovery of cell volume during mechanical strain is important for TM cell viability.

Our study of ANP signaling and RPE fluid transport indirectly suggest AQP1 could act as an ion channel in mediating solute and fluid transport. Further support for AQP1 as a cation channel in the RPE would be provided by the following studies. First, it would be necessary to measure currents across an RPE monolayer in the presence or absence of an AQP1 cation channel blocker to serve as preliminary evidence that the ion channel property is a potential contributor to RPE fluid movement. Second, patch clamp studies of single RPE cells in the presence of known agonists of an AQP1 current would also support a potential role for the channel. Third, characterization of other signaling mechanisms responsible for the effect of ANP on fluid transport would help determine how significant the effect of an AQP1 current may be. For example, if cGMP does indeed reduce intracellular calcium levels, it would be interesting to know what the effect is on net fluid transport and whether calcium dependent chloride channels are affected. Perhaps block of calcium release from stores would provide evidence of how significant

a calcium release might be for fluid transport. Fourth, further characterization of channels, such as the epithelial sodium channels (ENaC), that potentially contribute to cation flux across RPE would be beneficial in determining whether a contribution from AQP1 would be physiologically relevant.

The present data taken into account with information from past studies provides new perspective on the possible roles for AQP1 in ocular tissues. Also, these studies emphasize the importance of water movement through multiple tissue types and demonstrate how water movement may be essential for different reasons depending on the tissue being studied. Improved understanding of AQP1 roles and regulation can help provide new insights with respect to prevention and treatment of related diseases. Prevention of glaucoma for example is dependent in part upon regulation of fluid movement through the conventional outflow pathway. Disruption in this process due to degradation of the TM could lead to insufficient aqueous humor outflow and increased IOP, a hallmark of glaucoma. Manipulation of AQP1 function in the TM during mechanical strain may provide a new avenue for stabilizing the TM and reducing damage associated with pathological conditions such as glaucoma. Similarly, the new characterization of ANP signaling in the RPE and resultant effects on RPE fluid transport indicate that AQP1 function can be regulated through different means and can support both traditional and non traditional roles. Investigation of ANP signaling has provided a new target for regulation of AQP1 function in RPE and new potential therapies associated with retinal edema or detachment. Combined the total study emphasizes how

water movement can serve multiple purposes and be regulated in a tissue specific manner.

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