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WATER AND ION TRANSPORT IN OCULAR TISSUES.

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Running Title: ***Epithelial Electrolyte and Fluid Transport***

Introduction

Ion-coupled fluid secretion (or absorption) is a property of epithelia, in general, and such fluid transport has been demonstrated with all ocular tissues examined to date, which include corneal endothelium and epithelium, and the epithelia of the ciliary body, lens, and conjunctiva, as well as, the retinal pigmented epithelium.

Corneal Endothelium

This mono-cellular layer is responsible for maintaining stromal hydration and hence, transparency. Although a theoretical understanding of the mechanism(s) by which the corneal endothelium produces ion-coupled fluid secretion has not been firmly established, it appears that corneal deturgescence depends on a vectorial, transcellular Cl^- and HCO_3^- transport in the stromal-to-aqueous direction across the endothelium. For further information on this subject the reader is referred to a recent review by Bonanno (2003).

Ciliary Epithelium

Besides the corneal endothelium, the ciliary epithelium is also widely recognized for its fluid transporting capability. Its function has been described in detail in a recent review by To *et al.* (2002). When compared against the amounts of fluid absorbed across the human proximal renal tubule, or across the small intestinal epithelium (180 liters per day and 9 liters per day, respectively), the amounts of fluid transported by eye tissues may seem meager (e.g., 0.004 liters per day is thought to cross the ciliary epithelium). Yet, besides the obvious cases of the corneal endothelium and ciliary epithelium, the fluid transported by other eye tissues that have been studied may also serve important functions, as discussed below.

Corneal Epithelium

Active chloride transport across the corneal epithelium in the stromal-to-tear direction was first described by Zadunaisky (1966) in the frog. Subsequently, the corneal

epithelium served as a classical model for Cl^--secreting epithelia whereby the basolateral membrane uptakes Cl^- into the cell via a Na^+-K^+-$2Cl^-$ cotransporter and releases the anion into the apical milieu via Cl^- channels (Candia, 1989) possibly including the CFTR. Basolateral Cl^- uptake does not depend upon the parallel activities of the Na^+/H^+ and Cl^-/HCO_3^- exchangers. In addition to the active transport of Cl^-, the frog corneal epithelium also exhibits a Na^+ absorptive activity in the opposite, tear-to-stromal direction representing 5-10 % of the net, transcellular transport of electrolytes across the epithelium. In the case of the rabbit corneal epithelium, the Cl^- secretory and Na^+ absorptive transport rates are nearly equivalent (Klyce and Crosson, 1985). To date, the electrogenic element(s) mediating Na^+ movement across the rabbit apical membrane has not been firmly established. Rabbit epithelial Na^+ transport is unaffected by amiloride, suggesting the absence of the major epithelial Na^+ channel (ENaC) that is commonly found in other Na^+-absorptive tissues. However, this channel has recently been detected by immunochemical analysis in the rodent and human cornea (Mirshahi et al., 1999), and there is functional evidence for its presence in the bovine corneal epithelium (Midelfart, 1987).

Given its Cl^- secretory mechanism, Klyce and Crosson (1985) noted that the epithelium has the potential to participate in the stromal-thinning process; a view seconded by Yang *et al.* (2000), who measured a spontaneous net fluid movement in the basolateral-to-apical direction across cultured rabbit corneal epithelial cells due to the osmotic gradient produced by endogenous transport activity.

The arguments for a possible contribution to deturgescence by the epithelium are limited, but plausible. Rates of transepithelial net fluid transport from stroma-to-tear (as measured by stromal thinning of corneas denuded of endothelium) are stimulated by Cl^- secretagogues (Klyce, 1977). Although the rate of stromal thinning mediated by the epithelium is many fold less (≈ 30 times; Klyce, 1975) than that produced by the endothelium, it is possible that *in vitro* measurements of transepithelial fluid movement do not represent the *in vivo* rate. Epithelial Cl^- transport is apparently under neurophysiological control and corneal innervation is severed in the *in vitro* protocols. It has been noted that the endogenous levels of cAMP decrease in the epithelium as function of time *in vitro* (Klyce, 1975). In addition, Yang *et al*. (2000) measured a fluid transport rate across corneal epithelial cells grown on permeable supports of $\approx 5\ \mu L \cdot hr^{-1} \cdot cm^{-2}$, or a rate comparable to that of other fluid transporting layers, such as cultured bovine corneal endothelial cells ($\approx 4\ \mu L \cdot hr^{-1} \cdot cm^{-2}$ obtained by Narula et al., 1992).

Implicit in the epithelial fluid transport studies cited above is the fact that the Cl^- transport rate must have exceeded the transapical Na^+-absorptive activity of the rabbit cornea in order to obtain epithelial-dependent stromal thinning, or transepithelial fluid secretion. In this regard, Klyce *et al*. (1973) reported that when Cl^- transport is stimulated by secretagogues, it represents 75 % of the transepithelial transport current. Furthermore, under open-circuit, a condition that reflects the *in vivo* situation, secretagogues elicit net Na^+ and Cl^- fluxes in the stromal-to-tear direction (Klyce, 1975). In short, there are indications that the corneal epithelium is capable of net fluid transport and may contribute (although many fold less than the endothelium) to stromal deturgescence.

Conjunctival Epithelium

Identical to other Cl^- secreting epithelia, the conjunctival epithelium (based on the rabbit model) has a basolateral bumetanide-sensitive Cl^- uptake process (mediated by the Na^+-K^+-2Cl^-cotransporter, NKCC1) positioned in series with apical Cl^- channels, including CFTR (Turner *et al.*, 2002). In addition, Na^+/H^+ and Cl^-/HCO_3^- exchangers exist in parallel in the basolateral membrane and can also mediate Cl^- uptake (Turner *et al.*, 2001).

An oppositely directed, electrogenic Na^+ reabsorption is amiloride-insensitive, and occurs via Na^+-dependent cotransporters such as those carrying glucose and amino acids (Hosoya *et al.,* 1996) in series with the basolaterally located Na^+-K^+ pump. Furthermore, non-selective cation channels (NSCC) have been identified in whole-cell patch clamping of freshly isolated conjunctival epithelial cells, and the possibility that such channels reside at the apical surface was suggested (Turner, *et al.*, 2000; **Fig. 1**).

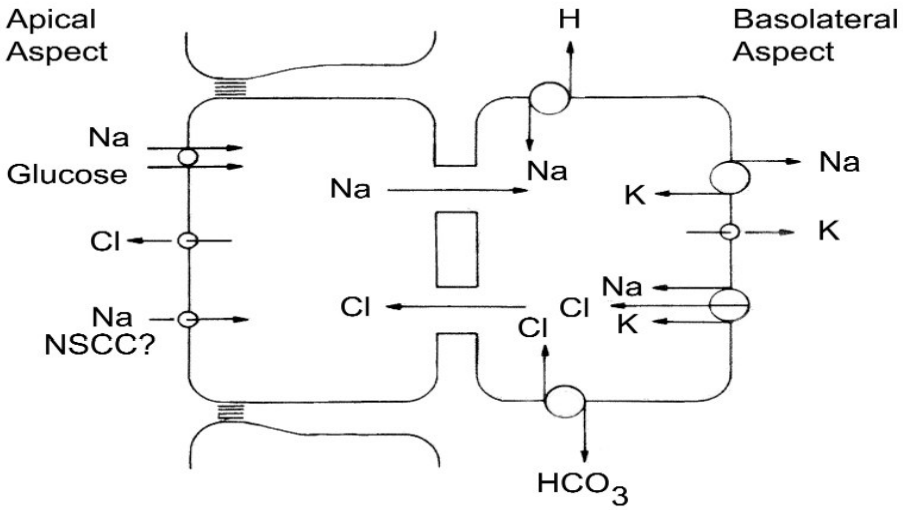

Fig. 1. *A cartoon summarizing the major transport elements that contribute towards transepithelial electrolyte movement across the conjunctiva.*

Spontaneous fluid transport across the conjunctival epithelium engenders a net flow in the basolateral-to-apical direction (Shiue *et al.,* 2000; Li *et al.,* 2001), a property consistent with the more dominant Cl^- secretory activity of the tissue. The reported fluid secretion rates were ≈ 4 $\mu L \cdot hr^{-1} \cdot cm^{-2}$ (Shiue *et al.,* 2000) and ≈ 6 $\mu L \cdot hr^{-1} \cdot cm^{-2}$ (Li *et al.,* 2001). These studies demonstrated that the measured fluid secretion was dependent upon transepithelial electrolyte transport. Both studies showed that the net fluid movement was increased (≈ 50 – 100 %) by Cl^- secretagogues that included purinergic agonists apparently acting via $P2Y_2$ receptors. Furthermore, based on the stimulatory effects of purinergics on the short-circuited epithelium, both studies also found that the increase in the transepithelial transport current correlated with the increase in fluid transport. This latter observation implies that $P2Y_2$ receptor activation leads to a selective stimulation of only the Cl^- secretory activity of the epithelium.

Diffusional water fluxes (J_{dw}) and mannitol fluxes were also measured across the rabbit conjunctival epithelium (Candia *et al.*, 1998). This approach indicated that the conjunctival mucosal surface is highly permeable to water and that the transepithelial water permeability (10^{-4} cm/sec) exceeded the paracellular permeability (10^{-6} cm/sec).

An early concept recognized two types of secretion by the secretory epithelia of the ocular surface and the various orbital glands – basic secretion and reflex secretion. Basic secretion was regarded as a baseline rate of production and reflex secretion as an increased rate caused by neuronal stimulation of the main lacrimal gland. Overall, the conjunctival epithelium has sufficient water permeability and the transporters necessary to contribute significant fluid to the tear film ($\approx 50\ \mu L \cdot hr^{-1}$ based upon its total surface area; Shiue *et al.,* 2000; Li *et al.,* 2001). This level of fluid flow is sufficiently large that it may represent the baseline tear secretion not attributed to the lacrimal gland. In addition, because purinergic agonists stimulate mucin secretion by conjunctival goblet cells, it seems plausible that the role of epithelial Cl^- transport includes the hydration of mucins upon release.

Intact Whole Lens and Lens Epithelia

The ocular lens is an avascular, quasi-spherical transparent organ comprised entirely of epithelial cells at various stages of differentiation. An epithelial monolayer extending from the anterior pole to the equatorial surface overlays the elongated, mature, enucleated cells, commonly referred to as the "lens fibers," that fill the bulk of the organ. A collagen capsule envelops the entire system and serves as the basement membrane for the epithelium, so that the apical face of the cells is oriented inward. The majority of homeostatic functions, such as the control of metabolic content, are mediated by the epithelium, the transport activities of which, affect the overall lens status due to the syncytial nature of the organ. The epithelial cells, while coupled to one another, are not functionally coupled to the fibers beneath at the anterior pole, with the interface between the fibers and epithelium constituting a virtual space. However, communicating junctions between the superficial and underlying cells are abundant at the equatorial region (Zampighi *et al.,* 2000), where the morphology resembles that of a multi-layered epithelium. Within the cortex, the fiber cells are highly coupled to each other.

Some early investigations emphasized the influence of the lens epithelium in conferring to the lens asymmetrical electrophysiological properties (Candia *et al.,* 1970). When isolated in a double chamber that separated the anterior from the posterior lens surfaces, an anteriorly directed, positive electrical potential developed, in all studied species, as a result of an electrogenic Na^+-K^+ pump located in the surface, basolateral membrane of the epithelium, along with parallel basolateral K^+ conductance(s). Logically, it was assumed that the activity of this monolayer was relatively uniform, and that a positive current emanated from the anterior pole to the equator. However, Patterson and coworkers (Robinson and Patterson, 1982-83) using the vibrating probe, showed in frog and rat lenses a non-homogenous distribution of currents around the lens surface.

More recently, Candia and Zamudio (2002) designed a novel Ussing-type chamber that enabled the short-circuiting approach to be used to characterize the regional distribution of Na^+ and K^+ currents around the surface of the rabbit lens. A non-uniform current distribution was found in the epithelium from the anterior pole to the equator due to

the absence of the Na^+-K^+ pump-generated current at the anterior polar region, where passive inflow of Na^+ occurred, and the dominance of K^+ conductances plus the pump current at the equatorial surface. As such, this work represented the first independent confirmation of the findings of Patterson and coworkers, as well as provided the identification of an additional asymmetrical aspect in the lens, namely the absence of Na^+-K^+ pump-generated current at the anterior-most surface.

Thus, the lens is an asymmetrical organ, both structurally and functionally, with localized transport properties. The recognized asymmetrical nature of the lens provided the underpinning for the microcirculation model of Mathias *et al.* (1997) for fluid movement within the lens. They proposed that epithelial electrolyte transport leads not only to the observed currents circulating around the lens, but that such ionic transport provides the driving force for the intralenticular circulation of nutrients and metabolites due to a hypothetical fluid entry across the polar regions, followed by fluid circulation within the lens and equatorial exit.

Presently, there are only two published studies that attempted to directly detect fluid transport by the lens, and antithetical results were obtained. Fowlks (1973) qualitatively ascertained that fluid moved across the rabbit lens in the posterior-to-anterior direction, while Fischbarg *et al*. (1999) quantified a net fluid movement of ≈10 $\mu L \cdot hr^{-1} \cdot cm^{-2}$ across the rabbit lens in the opposite direction. The latter study also measured net fluid movement across cultured mouse and bovine epithelial cells in the basolateral-to-apical direction (≈ 3 $\mu L \cdot hr^{-1} \cdot cm^{-2}$ and ≈ 5 $\mu L \cdot hr^{-1} \cdot cm^{-2}$, respectively), thereby obtaining results consistent with the direction of the net flow that they determined across the intact lens. With the intact lens, these authors isolated the lenses within a chamber that occluded the equatorial surface, and speculated that the magnitudes of the circulating currents are insufficient to elicit substantial equatorial fluid efflux. This is contrary to the Mathias *et al.* model (1997) and to the findings of Candia and Zamudio (2002a) for substantial cationic currents at the equatorial zone, which are larger than at other areas of the lens surface **(Fig. 2)**.

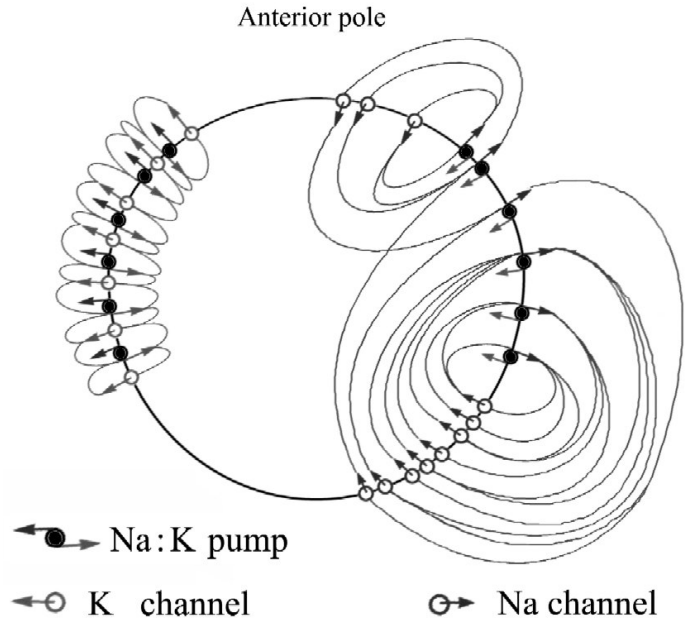

Fig. 2. *Schematic of the areas of exit and entry of $Na^+$and K^+ across the anterior, equatorial and posterior surfaces of the rabbit lens based upon the analysis of Candia and Zamudio (2002). The Na^+-K^+ pump-current activity is concentrated in the equatorial epithelium. For simplicity, epithelial K^+ channels and are shown on the left side, while the Na^+ channels are depicted on the right in the more anterior epithelium and posterior surface. Na^+ influx occurs at the anterior polar region and the entire posterior face and effluxes primarily via the equator. The internal circulation of K^+ may be more localized because diffusional currents and Na^+-K^+ pumps overlap in their distribution. The surface expression of Cl^- channels has not been characterized; uniformity about the entire lens is presumed.*

Subsequently, Candia and Gerometta (2003) presented a new chamber design **(Fig. 3)** to empirically measure fluid movement across the relatively large freshly isolated bovine lens. For this approach, the lenses were isolated within the chamber with 2 O-rings separating the lens semi-spherical surface into 3 regions: anterior pole (8-mm diameter), epithelial-equatorial, and posterior face. The chamber was closed except for a connection from each of the 3 compartments to its own capillary, which was used to measure volume changes. Changes in one capillary are compensated by opposite changes in the other capillaries, so that the volume in the system is constant. Only results from experiments that showed no external or internal leaks were considered. Furthermore, only those in which the algebraic sum across the 3 designated areas were close to zero were considered. The empirical measurements determined (using additional data obtained since the submission of the Candia and Gerometta 2003 abstract) that fluid movement across the anterior surface area was inward (≈ 1.84 μL per hr), whereas flows across the equatorial and posterior surfaces were outward (1.60 and 0.29 μL per hr, respectively). These observations are not totally consistent with the widely held theoretical model (Mathias *et al.,* 1997) that predicted net inward fluid flows at the poles and efflux at the equator, based upon observed currents circulating around the lens. However, the driving forces for the observed fluid movement in the bovine lens are unknown and probably differ from other more widely characterized mammalian lenses (e.g., rabbit). Further work will be needed to determine the surface distribution of electrolyte and solute transporting elements about the bovine lens, as well as, determine the possible influence of secretagogues on lens fluid transport.

An additional aspect of fluid movement within the lens may involve the rapid re-equilibration of fluid that might occur under the mechanical stress of changes in lens shape during accommodation. Presently, this possibility has not been addressed because traditional theories suggest that lens volume is constant during accommodative changes.

Briefly, the lens adopts a more rounded shape when an individual focuses on a short distance and a thinner more flattened shape with a larger equatorial diameter for focusing on infinite distance. These changes in shape must be accompanied by changes in capsular surface area, lens volume, or both. Because the lens capsule is very stiff and can be folded, but is difficult to stretch, and because the lens is capable of gaining or losing fluid when exposed to anisotonic solutions, we recently considered the possibility that during accommodative changes the lens also gains or loses fluid.

Consistent with this idea, a recent MRI study found that the human lens cross-sectional area (CSA) increased during the accommodation for near vision and decreased for infinite focusing (Strenk *et al.*, 2004). Although these authors recognized that the changes in CSA reflected changes in volume, they assumed that the lens material was compressible, and did not consider the possibility that fluid could have left the lens when its CSA was decreased.

An initial examination was begun (Candia *et al*., 2005) to measure the amount of fluid that could move out of and into the bovine lens. Compression of the lens under oil resulted in a loss of lens mass that was restored by submerging the lens within an aqueous physiological solution. Thus fluid left the lens and re-entered secondary to changes in lens shape. We also developed theoretical methods to calculate that the lens volume is proportional to CSA. Changes in lens CSA similar to that occurring during accommodation result in calculated changes in lens volume.

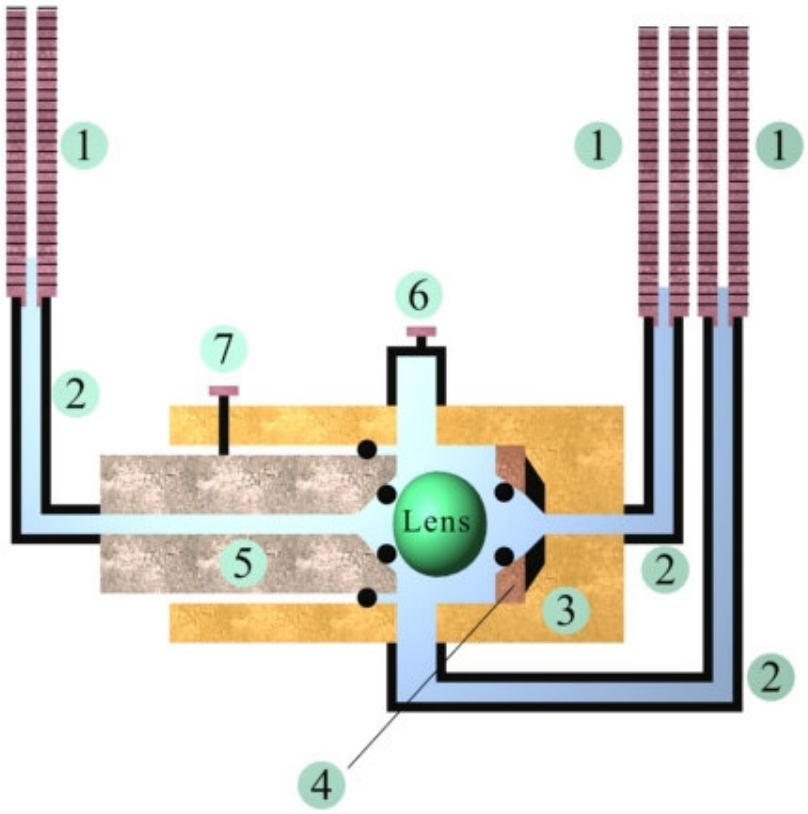

Fig. 3. Schematic of the chamber used by Candia and Gerometta (2003) to measure fluid movement across the bovine lens.

1) 100µl Hamilton syringes with 1µl divisions. 2) Leak-proof Luer® connectors. 3) Body of the Lucite® chamber with cylindrical cavity to accommodate piston 5. 4) Washer (1 of 3) that can accommodate O-rings of different diameters to adapt to the size of the lens. It lays flat against the bottom of the cylinder held by a layer of grease. 5) Piston that is introduced until the central O-ring touches the lens and seals the center (equatorial) compartment from the anterior compartment. Piston is made with different sizes of bed to accommodate 3 different central O-rings to adapt to the size of the lens, and to include different areas of anterior surface around the anterior pole. An outer O-ring seals the piston to the chamber cylinder and allows for its controlled displacement. 6) Bleeding outlet to release fluid as piston is introduced. It is closed after final adjustments are made. By applying a slight pressure with a piston to any one of the Hamilton syringes, one can determine if the 3 compartments are isolated by checking the levels of the other 2 syringes. 7) Screw to hold piston in place after all adjustments are made.

From this, we propose that the natural lens has an important physical property necessary for accommodation, namely a high content of fluid that can redistribute among

the internal aquaporin 0-rich fibers **(see Fig. 4)**, and permeate the capsule, thereby resulting in a net exit and entry of fluid during the accommodative cycle.

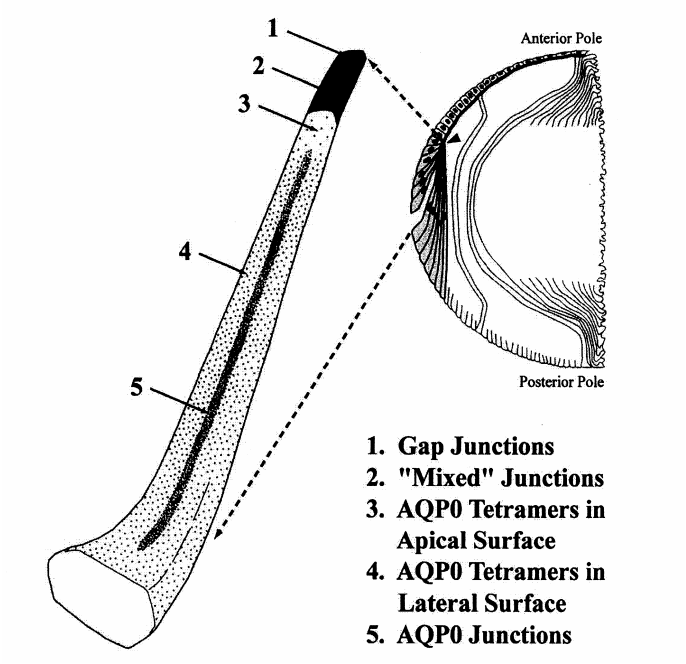

Fig. 4. *A diagram of the distribution of aquaporin 0 in the lens equatorial fiber that was copied from Zampighi et al. (2002).*

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