Ion transport mechanisms in the formation of aqueous humor

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Abstract

The aqueous humor dynamics of the eye are frequently associated with the vision-threatening disease, glaucoma. Pharmacological treatment for glaucoma generally aims to lower the intraocular pressure by reducing aqueous humor formation. Although the aqueous humor is believed to be actively secreted by the ciliary epithelium, the mechanism is still poorly understood. Many studies have been carried out in the last 40 years to unravel the underlying ionic mechanisms that drive aqueous humor formation. This review briefly surveys the ion transports in the ciliary epithelium, such as the possible roles of sodium, chloride and bicarbonate ions as the driving forces. It also examines the current ionic models for aqueous humor formation and its regulation from a cellular transport perspective.

Key words: *Ciliary epithelium, Ion transport, Aqueous humor formation*

Introduction

The aqueous humor of the eye is produced by the ciliary epithelium and is drained at the anterior chamber angle. The rate of aqueous humor formation in the human eye is about 120 µL per hour. The dynamic balance between the aqueous inflow and outflow produces the intraocular pressure (IOP). Apart from its role in providing nutritional and structural support for the optical components of the eye, the IOP is frequently associated with a serious eye disease – glaucoma.

Of the different types of glaucoma, primary open-angle glaucoma is the most common and most serious because of its insidious nature.

Primary open-angle glaucoma is an optic neuropathy which is characterized by progressive damage in the optic nerve fibers and characteristic visual field losses. It is also frequently associated with an elevation of IOP. According to the World Health Organization (WHO) criteria for blindness, glaucoma is the third most common cause of blindness in the world.[1] It is estimated that there are currently 3 million people blind from glaucoma worldwide.[1] Primary open-angle glaucoma is also the second or third most common cause of blindness in the developed countries like the United States and the United Kingdom.[2] Although statistics on glaucoma sufferers in the Chinese population is limited, it is believed that glaucoma is among the leading causes of blindness in China as well.[3]

Increases in the IOP are due to an imbalance between the inflow and outflow of the aqueous humor. It is generally thought that blockage of the anterior drainage causes the elevated IOP.[4] However, the main pharmacological antiglaucoma treatment still involves lowering the IOP by decreasing aqueous humor production. It is therefore surprising that the basic physiological mechanism of aqueous humor formation by the ciliary epithelium, let alone its regulation, is still poorly understood.

The ciliary epithelium and ion transport

Although the mechanism of aqueous humor formation was once thought to be via passive processes such as diffusion

and ultrafiltration, it is now widely believed that active secretion by the ciliary epithelium is the dominant mechanism.[5,6]

The ciliary epithelium acts as a selective barrier to various substances,[7] and is composed of two layers: the nonpigmented (NPE) and the pigmented epithelium (PE). The two epithelia are aligned in an apex-to-apex fashion, with the NPE forming the external boundary of the tissue and facing the aqueous side. The presence of tight junctions at the apices of the NPE membrane effectively blocks off paracellular diffusion of various solutes and ions. Therefore, most of the ions and metabolites will take a transcellular route from the stroma of the ciliary body to reach the posterior chamber of the eye. In terms of aqueous humor formation, the ciliary epithelium actively pumps solutes or ions to the aqueous side and creates concentration gradients. The gradients then provide a force to drive the bulk flow of water into the eye; this constitutes the aqueous inflow macroscopically. It is therefore imperative in the study of the aqueous humor formation, firstly, to characterize the key transcellular active solute or ion pumps that power the fluid flow (transepithelial transport), and secondly, to identify the membrane components that orchestrate the solute or ion pumps (transmembrane transport).

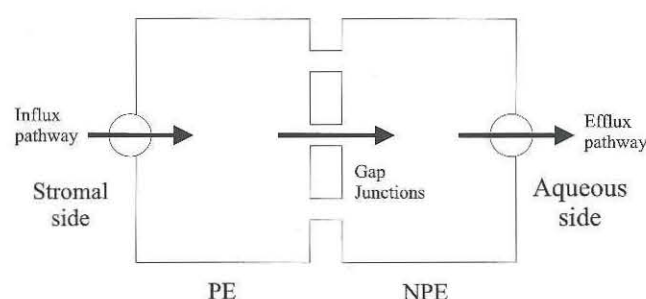# The ciliary epithelium is a functional syncytium

Figure 1. Influx and efflux pathways for ions and solutes to go through the bilayer ciliary epithelium. Ions and solutes must go through the basolateral membrane of the pigmented epithelium (PE) first, then through the gap junctions between the two cell layers, and finally exit through the nonpigmented epithelium (NPE). In effect, ions and solutes are transported from the stromal to the aqueous side.

One of the characteristic features of epithelia is the asymmetry of their membrane transporters, which is essential for carrying out vectorial transport. Transepithelial transport consists of at least two transmembrane transport steps: influx of the ions or solutes into the cell and efflux of the ions or solutes across the opposite membrane. It was previously thought that the NPE cell played the predominant role in aqueous secretion, since the tight junctions are found only between the NPE cells.[8] Wiederholt et al suggested a functional syncytium model to explain the role of the PE and NPE cells according to their different physiological properties.[9] The asymmetric transport properties provide division of activities (Figure 1): ions and solutes are firstly taken up from the blood side by the PE cells. They are then

transported or diffused through the gap junctions between the two epithelial cells[8] and can be accumulated inside the NPE cells. Driven by the electrochemical gradients and/or active transport, ions and solutes diffuse and/or are pumped out from the NPE cells. The net effect is the transfer of ions and solutes from the stromal to the aqueous side. This functional syncytium hypothesis is supported by the observation of free dye diffusion between paired PE and NPE cells after microinjection of Lucifer yellow.[10] Further evidence has come from intracellular recordings of the PE and NPE layers. It has been found that the intracellular potential is similar in both epithelial layers, suggesting that both cell layers are electrically shunted.[11]

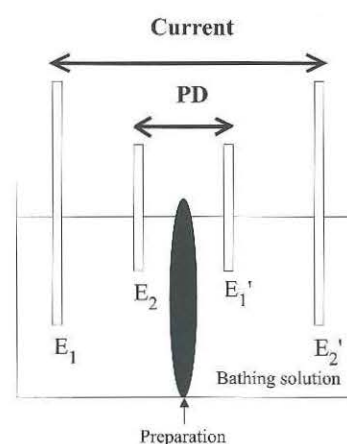# Electrophysiology of *in vitro* iris-ciliary body epithelium preparations

Figure 2. A schematic diagram showing the basic electrical measurements with an Ussing-type chamber. A preparation is bathed in two separate half chambers with identical solution. A potential difference (PD) is developed across the preparation as a result of all ion tranport. The PD can be clamped to zero by a counter-current (short-circuit current). This current is equivalent to the net transfer fo charge due to active transport. The integrity of the preparation can also be determined by measuring the transtissue electrical resistance. E_1E_1' and E_2E_2' are two pairs of electrodes for measuring the PD and short-circuit current, respectively.

Transepithelial transport can be studied with an Ussing-Zerahn-type chamber, where the basolateral and apical sides of the epithelium are separated and facing isolated bathing solutions (Figure 2).[12] Epithelial preparations often generate standing electrical signals in the form of potential difference (PD), current and resistance. These parameters reflect the resultant of all transport activities of epithelial preparations, and therefore, they have been extensively studied in ciliary epithelium preparations by many researchers. Early study by Cole showed that the PD of the iris-ciliary bodies of the ox and the rabbit were positive at the aqueous side.[13] It was postulated that active cationic transport was involved, and sodium was apparently the responsible ion. However, more recent electrophysiological studies on the ciliary epithelium of the rabbit,[14-16] cat,[17] dog,[18] and toad[19] have shown a negative PD at the aqueous side. These results pointed to an active anionic rather than cationic transport. It was suggested that chloride or/and bicarbonate ions were involved in the

active transport process. Substituting these ions in the bathing solution and studying their effect on the preparation may reveal the relative importance of these ions on aqueous humor secretion. Removing bathing bicarbonate reversed the PD across the rabbit ciliary epithelium,[14,15] making the aqueous side positive relative to the stromal side. Although not reversing the polarity of the standing PD, bicarbonate-free solution depolarized our bovine ciliary epithelium preparation.[20] Therefore, it is apparent that bicarbonate plays an important role in the active transport mechanism. The role of chloride is controversial. Studies on rabbit ciliary epithelium have demonstrated the dependence of PD on the bathing chloride concentration,[15] while others have found no significant change in PD with low levels of bathing chloride.[14] We have recently shown a clear dependence on chloride concentration in bovine ciliary epithelium preparations: the PD changed dose-dependently with bathing chloride concentration.[20] With low chloride concentration (30 mM), the PD was reversed in polarity (unpublished observation). Interestingly, this response is very similar to that found with rabbit ciliary epithelium bathed in bicarbonate-free solution.[14] One of the reasons for the discrepancy can be attributed to species difference, but one may need to establish the viability of this very fragile preparation before drawing any conclusion. Although ion depletion experiments have provided good insights into the global view of the transport activities in the ciliary epithelium, they have not addressed the key question, that is, which is or are the ions that take part in transepithelial ion transport. In order to elucidate the involvement of particular ions, flux studies using radioisotopes can provide the missing link.

Transepithelial ion and substrate transport

Substrate transport

Net ascorbate flux: Kinsey and Reiss *et al* have shown that the ascorbic acid concentration of the aqueous is much higher than that of the plasma.[21,22] One of the functions of ascorbate is thought to be antioxidation protection for the anterior eye.[23] The literature generally postulates that ascorbate is actively transported into the aqueous humor. Helbig *et al* reported that ascorbate was accumulated intracellularly in cultured bovine PE.[23] Most directly, Chu and Candia, and Mok and To have demonstrated a net transepithelial ascorbate flux to the aqueous side across the rabbit and bovine ciliary epithelia in an Ussing-type chamber.[24,25] Apparently, the rabbit ciliary epithelium transports ascorbate into the aqueous at a much faster rate than that in the ox. The reason for this discrepancy is unclear, but the ascorbate concentrations in the aqueous humor are different in different mammalian species.[22] This is likely to be related to differences in active ascorbate transport activities.[26] Although the active ascorbate transport can, in theory, contribute to the driving force for aqueous humor formation, the fluid produced by ascorbate transport is far too little to account for aqueous humor formation.[25]

Glucose transport mechanism

Glucose supply to the lens, cornea and anterior tissues of the eye is via the ciliary body and ciliary epithelium. Glucose is stereospecifically transported across the ciliary epithelium, with the D-glucose analog being transported twice as fast as the L-glucose, and no net glucose transport can be detected.[25] It has been suggested that D-glucose transport is via a bidirectional carrier-facilitated diffusion mechanism across the ciliary epithelium or ciliary body. Immunohistochemical investigation has identified the GLUT1 glucose transporter in the ciliary epithelium that is responsible for passive facilitation of glucose transport.[27]

Ion transport

Chloride transport

Net chloride ion fluxes towards the aqueous side have been reported in the cat,[17] the toad,[28] the rabbit,[29] and recently, in the ox.[30] Pesin and Candia observed an apparent net chloride ion flux into the posterior chamber.[31] However, the unidirectional fluxes were variable and the net chloride transport was therefore rendered statistically insignificant. Interestingly, the net chloride transport was frequently many times higher than was expected from the measured current (which is a summation of all ion transport activity).[29,30] One likely reason may be that the chloride transport is electrically coupled to other cationic transport (that is, of sodium ions) in the same direction and renders the flow of ions electrically silent. Typically, in epithelia where net sodium or/and chloride ion fluxes have been found, the unidirectional ion flux is usually two or more times higher than the opposite flux.[32,33] However, this was not the case in the ciliary epithelium study. Candia *et al* detected a statistically significant net sodium ion flux with low bathing sodium; the inward flux was about 20% greater than the outward flux.[34] We found that the inward chloride flux was about 20% larger than the opposite flux.[30] This could be easily explained by extremely leaky tissue with high paracellular permeability. However, we have proposed that the isolated bovine ciliary epithelium and ciliary body may not be as leaky as has been thought.[25,30] Since the ciliary body is a heavily folded structure, it has been estimated that the true surface area might be as high as sixfold its apparent area, which corresponds to a resistance of about 500 Ωcm^2.[6] In addition, the low L-glucose diffusion across the bovine ciliary body and epithelium[25,30] was found to be comparable to diffusion across a typical tight epithelium.[32] Thus, the contribution of passive diffusion through the paracellular pathways and edge damage to the unidirectional fluxes should be small. Strong bidirectional electroneutral Na-Cl cotransport across the ciliary epithelium[35] may explain the small difference between the two unidirectional chloride ion fluxes. Such bidirectional transport of ions will also support the hypothesis that the ciliary epithelium performs reabsorption as well as secretion functions.[36]

Bicarbonate transport

The role of bicarbonate in the aqueous humor formation has been extensively examined because of the effect of carbonic

anhydrase inhibitor in lowering the IOP.[37] Riley and Kishida have found bicarbonate-stimulated ATPase in the bovine ciliary epithelium.[38] Although Kishida et al,[15] Krupin et al[14] and Murakami et al[39] have shown the effect of bicarbonate ion on the electrophysiology of the ciliary epithelium, no conclusive bicarbonate net flux has been reported so far. Candia recently described a new method, which can accurately measure the ^{14}C-labeled fluxes of HCO_3^- and/or CO_2 across epithelia.[40] He suggested that in his system the specific activities of HCO_3^- and CO_2 were equal in the bathing solution in the half chambers. Using the same working hypothesis, we measured the unidirectional bicarbonate ion fluxes across the bovine ciliary epithelium and ciliary body. No net bicarbonate flux was found.[41] Although bicarbonate ion may not be involved in the transepithelial transport, it may modulate the transmembrane ion transports which are crucial in the active secretion of aqueous humor, for example, exchange with chloride by protein exchange (to be discussed below).

Sodium transport

Sodium ion transport may be involved in the active pump[13,42] and it may function by coupling with anion transport, as described earlier. Na,K-ATPases have been localized to both the PE and NPE cells.[43,44] Observations on the short-circuit current with ouabain have strongly suggested the presence of Na,K-ATPases pumping against each other at the two epithelial layers.[14,15,18,45] Histochemical studies have shown that there are more Na,K-ATPases in bovine NPE cells than in the PE cells.[38] Net sodium flux can therefore result from the difference between the activities of these two populations of Na,K-ATPases.

Many have attempted but failed to find any net sodium transport across the ciliary epithelium using Ussing-type chambers.[29-31] Saito and Watanabe detected a net sodium flux from stroma to aqueous, but it was too variable and therefore statistically insignificant.[28] Candia et al postulated that there may be a large diffusional bidirectional Na-Cl cotransport, which has rendered the detection of net sodium transport difficult.[34] They repeated the experiment at low sodium concentration (30 mM) to minimize the passive components. A net sodium ion flux in stroma to the aqueous direction was observed. Therefore, although no significant net sodium ion flux was found in normal physiological conditions, given the large background or unidirectional flux of sodium, a small active sodium transport in vivo cannot be ruled out.

The roles of ion transporters and channels

For transepithelial ion transport to take place, ions are required to traverse the bilayer ciliary epithelium. They need to go through the PE (influx pathway) first and be released into the eye via the NPE (efflux pathway) at specific ion transporters or channels.

Transmembrane transport: the influx pathway at the PE cells

The PE cell has been postulated to be the site of solute and water uptake mediated by multiple Na-dependent uptake mechanisms,[9] with Na,K-ATPase supplying the required energy gradient. Currently two major influx pathways of NaCl have been proposed: (i) the Na^+/H^+ antiport and Cl^-/HCO_3^- exchanger; (ii) the Na-K-2Cl cotransporter (**Figure 3**).
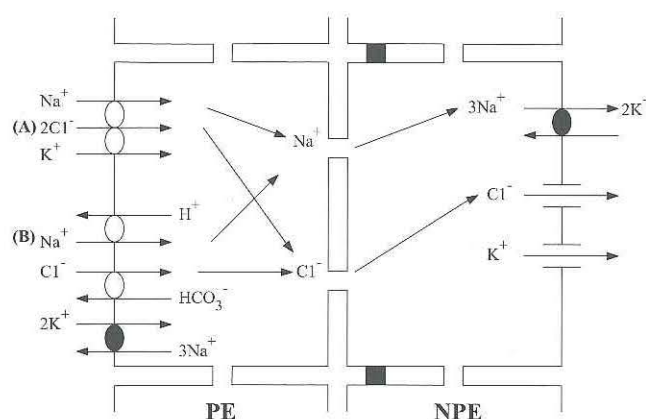

Figure 3. A simplified schematic diagram illustrating the possible pathways of chloride transport across the ciliary epithelium. (A) Electroneutral Na-K-2Cl cotransport and (B) Cl^-/HCO_3^- and Na^+/H^+ double exchangers. Both pathways allow the uptake of Na^+ and Cl^- into the functional syncytium in an electroneutral manner. The energy for the accumulation of chloride in pigmented epithelium is provided by the transmembrane Na^+ gradient generated by the Na,K-ATPase. The ions are subsequently diffusing through the gap junctions and flow out through the transporters and channels.

Na,K-ATPases

The role of the Na,K-ATPases in the PE is thought to be maintaining the transmembrane sodium and potassium ion gradients ('housekeeping' ATPase) rather than transporting sodium to the stroma.[9] The standing sodium ion gradient can provide energy for other ions to move into the PE cell against their electrochemical gradients. In cultured PE cells, many sodium-coupled ion transport mechanisms have been proposed.

Na^+/H^+ antiport and Cl^-/HCO_3^- exchanger

Both Cl^-/HCO_3^- and Na^+/H^+ exchangers have been identified in cultured bovine PE cells. The Cl^-/HCO_3^- exchanger may be responsible for chloride ion entry into the PE,[46] whereas the Na^+/H^+ exchanger can mediate sodium entry into the PE.[47] The exchangers are primarily physically independent, but physiologically they are coupled via carbonic anhydrase. Helbig et al have postulated a possible model for NaCl transport in the ciliary epithelium, which includes two forms of the carbonic anhydrase (CA II and CA IV) (**Figure 4**).[48] Membrane-bound carbonic anhydrase (CA IV) dehydrates extracellular carbonic acid to carbon dioxide and water, and this carbon dioxide diffuses into the PE cell through the cell membrane. Cellular metabolism probably provides another source of intracellular carbon dioxide. Intracellular carbonic

anhydrase (CA II) hydrates carbon dioxide, providing HCO_3^- and H^+ as substrates for Na^+/H^+ and Cl^-/HCO_3^- exchangers at the basal side of the PE. Consequently, NaCl is taken up by the PE cell, and HCO_3^- and H^+ are exported and once again recycled to carbon dioxide by membrane-bound carbonic anhydrase (CA IV). In this model, HCO_3^- plays a central role and a net transepithelial HCO_3^- transport is not required. McLaughlin et al recently showed evidence of chloride accumulation by these two exchangers in the rabbit PE cells and proposed that it is the chief ion uptake pathway.[49] However, when we tried blocking the Cl^-/HCO_3^- exchanger with its blocker, DIDS (4,4'-diisothiocyanatostilbene-2-2'-disulfonic acid), in a bovine ciliary epithelial preparation, the net chloride flux was not affected.[50] Although the exact reason for this discrepancy is unknown, species difference is a possibility, since we have observed clear differences in the response to bicarbonate-free solution between porcine and bovine ciliary epithelial preparations (unpublished observations).
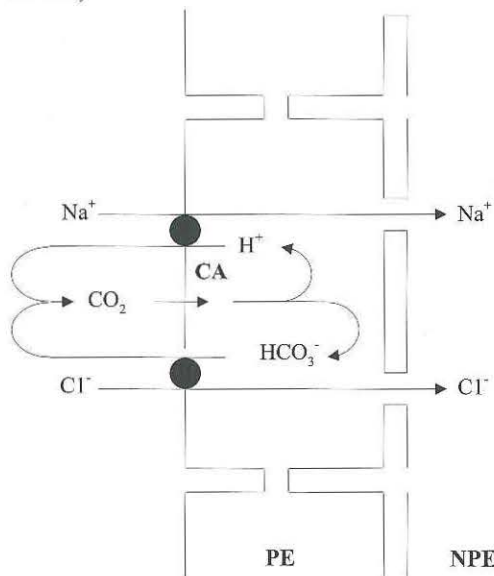

Figure 4. Influx pathway for Na^+ and Cl^- coupled with carbonic anhydrases at the pigmented epithelium.

Na-K-2Cl cotransport

Edelman et al found that PE cells possess a bumetanide-sensitive uptake mechanism, suggesting the presence of an Na-K-2Cl transport mechanism.[10] This is further supported by the observation of a bumetanide-sensitive ^{86}Rb uptake component in PE, which requires the presence of extracellular sodium and chloride ions.[51] We have also shown that the transepithelial chloride transport is inhibitable by bumetanide.[30,50] It is suggested that the Na-K-2Cl may play a significant role in chloride uptake at the PE.

Transmembrane transport: the efflux pathway at the NPE cells

The NPE cell is the putative site of solute and water efflux into the posterior aqueous chamber,[9] and the mechanisms may be mediated by the Na,K-ATPase and ion channels (Figure 3).

Na,K-ATPases

It has been suggested that Na,K-ATPases are functionally different in the PE and the NPE. Ghosh et al found that three α and two β isoforms of Na,K-ATPase are differentially expressed in the PE and NPE cell layers.[52] Na,K-ATPase has been localized to the basolateral membrane of NPE cells,[53] and as in the PE, it provides the primary energy gradient for other transmembrane transports to take place. Furthermore, this gradient may form the efflux pathway of the sodium into the eye, if indeed an active sodium transport is present in an in vivo situation.[34]

Chloride exit and chloride channels

Chloride ions leave the NPE cells and pass into the posterior chamber according to the electrochemical gradient,[9] most likely via chloride channels. Edelman et al identified a chloride-selective channel at the NPE.[54] They proposed that the sodium ion is exported by the Na,K-ATPase and is probably coupled with chloride ions. Chloride ions may leave the NPE via the cAMP-dependent chloride channels. The driving force for chloride exit can be further enhanced by membrane hyperpolarization, for example, increased potassium efflux via the calcium-dependent maxi-potassium channels.[54] The chloride net flux in bovine ciliary epithelium has been found to be very sensitive to a chloride-channel blocker (NPPB—5-nitro-2-(3-phenylpropylamino) benzoic acid) at the NPE side.[50]

Potassium channels

Because of the active pumping by the Na,K-ATPase, the intracellular potassium concentration is high. Potassium is thought to leave passively and be recycled through the various potassium channels in the NPE cells.[54,55]

Na-ascorbate transport

Helbig et al found that the bovine PE cells accumulated reduced ascorbate to about 40 times its bathing concentration,[23] and the accumulation was dependent on the extracellular sodium concentration. This finding suggests the presence of Na-ascorbate cotransport in the PE cells. Differences have been shown in the mechanisms of ascorbate influx at the PE cell and efflux at the NPE cell. In contrast to the active influx process in PE, ascorbate efflux has been shown to be neither energy-dependent nor coupled with other ions (such as sodium ion) at the NPE.[56]

In short, the sodium exit is likely to be via Na,K-ATPase, whereas potassium diffuses down its electrochemical gradient via potassium channels. Chloride efflux is by chloride channels (Figure 3).

Effects of pharmacological agents on active ion transport

The regulation of the aqueous humor formation by known pharmacological agents is likely to affect the ion transport mechanisms of the ciliary epithelium. The interaction

between these agents and various ion transport components is exemplified by the following drugs: furosemide, acetazolamide and timolol.

Furosemide

Furosemide (4-chloro-*N*-furfuryl-5-sulfamoyl anthranilic acid) is a fast and potent loop diuretic. The inhibitory action of furosemide on the active chloride transport mechanism has been documented in a variety of tissues, including the diluting segment of rabbit renal tubule[57] and frog corneal epithelium.[58] Furosemide also inhibits the bicarbonate and chloride exchanger in dog pancreatic duct[59] and in red blood cell.[60] In addition, furosemide has been shown to reduce the short-circuit current of isolated ciliary epithelium in the shark,[61] rabbit,[15,31] dog,[18] and toad.[62] Furosemide was also found to abolish the net chloride transport across the bovine ciliary epithelium.[30] These results suggest the presence of a furosemide-sensitive anion transport mechanism in the ciliary epithelium.

Acetazolamide

Carbonic anhydrase inhibitors, such as acetazolamide (N-[5-sulfamoyl-1,3,4-thiadiazol-2-yl]acetamide), have been clinically used to lower the intraocular pressure in glaucoma patients for over 30 years. It is believed that the carbonic anhydrase inhibitor inhibits carbonic anhydrase in the ciliary epithelium.[37] Carbonic anhydrase inhibitors have been shown to diminish unidirectional bicarbonate ion fluxes from blood to aqueous.[63] The aqueous-negative transepithelial potential difference in *in vitro* ciliary epithelium has been shown to depend on the presence of bicarbonate ions in the bathing solution.[14,15,39] Acetazolamide can also depolarize the PD across the ciliary body preparation.[39] Wiederholt *et al* have suggested that the role of carbonic anhydrase is to facilitate the sodium and chloride influx via the Na-H and Cl$^-$/HCO$_3^-$ exchangers.[9]

Timolol

Timolol is a potent topical nonselective β-blocker, which lowers the IOP by decreasing the aqueous humor formation.[64] The ciliary epithelium is rich in β-receptors.[65] Timolol is thought to act on the receptors and lowers the intracellular cyclic AMP concentration of the ciliary epithelial cells.[66] Intracellular cAMP is an important second messenger, which modulates cellular functions, including the activities of transporters and channels. Crook and Riese have shown that cAMP stimulates the activity of Na-K-2Cl in fetal NPE cells.[67] Therefore, timolol may act by depressing the intracellular cAMP level, which decreases the ion transport through the Na-K-2Cl. The result is a decrease in the aqueous humor formation and IOP.

Future direction

Physiologically, it will be important to identify exactly how the transepithelial ion transport (both chloride and sodium transport) are constituted in terms of transmembrane transport. At present, the debate on the major influx pathway at the PE goes on as to whether it is via the Na$^+$/H$^-$ and Cl$^-$/HCO$_3^-$ exchangers[46,47,49] or by the Na-K-2Cl cotransporters.[30,50,68] Identification and localization of the transporters or channels will be very important in devising a correct model for aqueous humor formation.

In terms of the regulation of aqueous humor formation, it will be important to characterize the modulation of the putative transporters or channels in play. Apparently, the regulation is related to many second messenger systems, such as cAMP/adenylate cyclase, PKC or cGMP cascades.[69-71] Therefore, if these regulation pathways on ion transport are established, new and potent antiglaucoma drugs may be designed to produce a long-lasting hypotensive effect.

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