At the back of our eyes, photoreceptors capture light and convert it into electrical signals that we perceive in our brain as vision. Photoreceptor function is energy expensive, even more so than many other processes in the body. Furthermore, photoreceptor metabolism increases in the dark and releases more metabolic by-products (CO₂, lactic acid, and H₂O) into the photoreceptor extracellular space (SRS). The retinal pigment epithelium (RPE) maintains photoreceptor health by transporting these metabolic acids from the SRS to the choroidal blood supply. By using native and cultured fetal human RPE, we show that the apical membrane is significantly more permeable to CO₂ than the basolateral membrane. This feature traps CO₂ in the cell and drives carbonic anhydrase (CA)-mediated hydration of CO₂ into HCO₃⁻, which is subsequently transported out of the basolateral membrane by a Na⁺-linked HCO₃⁻ co-transporter (NBC). This process increases net steady-state fluid absorption, thus maintaining retinal adhesion to the RPE.

Oxidative metabolism generates significantly more ATP than glycolysis, but photoreceptors derive ≈ 50% of their total ATP consumed from glycolysis due to the low oxygen level at the photoreceptor inner segment. Furthermore, lactic acid production and release into the SRS almost doubles in the dark. We show that the RPE transports lactic acid from the SRS via a proton-linked monocarboxylate transporter (MCT1), and this process activates pH₁-regulatory mechanisms at the RPE apical membrane: Na⁺/H⁺ exchanger (NHE) and Na⁺-linked HCO₃⁻ transporters (NBC1 & NBC3). These mechanisms also facilitate MCT1-mediated lactic acid transport by preventing buildup of a proton-gradient across the RPE apical membrane.

We show that an increase in SRS CO₂ or lactic acid level causes RPE cell swelling. The RPE alleviates swell-induced osmotic stress by activating apical membrane K⁺-channel (Kir 7.1) and basolateral membrane Cl⁻-channel (ClC-2), which drives KCl (and fluid) out of the cell to decrease cell volume. In this study, we identified the cellular mechanisms in RPE that prevent acidosis and fluid accumulation in the SRS caused by increased photoreceptor metabolism in the dark. These homeostatic processes maintain the close anatomical relationship between photoreceptors and RPE, thus protecting photoreceptor health and preserving visual function.
Dedication

This work is dedicated to my Mom and Dad, Rusuanti and Winoto, to my brothers, Lawrence and Raymond, and to the love of my life, Melissa.
Acknowledgements

This dissertation will not be complete without properly thanking my advisors, Dr. Sheldon Miller, Dr. Sarah Sohraby, Dr. Nancy Philp, and Dr. Nam Sun Wang, whose guidance, patience, and excellent mentorship have taught me what it takes to be a scientist. Many colleagues contributed significantly to this project: Dr. Wan Qin helped perform microelectrode experiments; Dr. Rong Li performed protein detection and localization experiments; Tina Banzon performed fluid transport experiments. In addition, Jing Zhao is responsible for the maintenance of the RPE cell cultures used in virtually all experiments presented in this dissertation. I’ve had many ups and downs throughout the projects, but in those tough times, I received solace from my colleagues, who somewhat willingly listened to my rants and raves. Finally, I want to extend a special thanks to Dr. Miller, who provided me my second home in his laboratory, who taught me proper English and worked tirelessly on my manuscripts.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>(Ap)</td>
<td>apical (bath)</td>
</tr>
<tr>
<td>(Ba)</td>
<td>basal (bath)</td>
</tr>
<tr>
<td>ABCC1</td>
<td>multi-drug resistance protein 1</td>
</tr>
<tr>
<td>ace</td>
<td>acetate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AE</td>
<td>anion exchanger (e.g., AE1, AE2, AE3)</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AQP1</td>
<td>aquaporin 1 (water channel)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCECF</td>
<td>2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein</td>
</tr>
<tr>
<td>βh/HCO3</td>
<td>CO₂/HCO₃ buffering capacity</td>
</tr>
<tr>
<td>βi</td>
<td>intrinsic buffering capacity</td>
</tr>
<tr>
<td>βtotal</td>
<td>total intracellular buffering capacity</td>
</tr>
<tr>
<td>BZA</td>
<td>benzolamide</td>
</tr>
<tr>
<td>CA</td>
<td>carbonic anhydrase</td>
</tr>
<tr>
<td>CD147</td>
<td>MCT accessory protein (also called basigin)</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CIC-2</td>
<td>Chloride channel</td>
</tr>
<tr>
<td>CPE</td>
<td>choroid plexus epithelium</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DApical</td>
<td>diffusion coefficient of CO₂ across the apical membrane</td>
</tr>
<tr>
<td>DBasal</td>
<td>diffusion coefficient of CO₂ across the basolateral membrane</td>
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<td>DIDS</td>
<td>4,4'-Diisothiocyanatostilbene-2,2'-disulfonate</td>
</tr>
<tr>
<td>DZA</td>
<td>dorzolamide</td>
</tr>
<tr>
<td>EₐA</td>
<td>apical membrane net reversal potential</td>
</tr>
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<td>EₐB</td>
<td>basolateral membrane net reversal potential</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
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<td>gₐ</td>
<td>apical membrane net conductance</td>
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<td>HEPES</td>
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</tr>
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<td>hRPE</td>
<td>human fetal retinal pigment epithelium</td>
</tr>
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<td>HIF-1α</td>
<td>hypoxia inducible factor 1 alpha</td>
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<td>Iₛ</td>
<td>shunt current</td>
</tr>
<tr>
<td>JAM</td>
<td>junctional adhesion molecules</td>
</tr>
<tr>
<td>Jvₗ</td>
<td>steady-state fluid absorption rate</td>
</tr>
<tr>
<td>Kir 7.1</td>
<td>inwardly rectifying potassium channel isoform 7.1</td>
</tr>
<tr>
<td>Lac</td>
<td>lactate</td>
</tr>
<tr>
<td>MCT</td>
<td>monocarboxylate transporter (e.g., MCT1, MCT3, MCT4)</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium eagle (culture media)</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>mOsm</td>
<td>milliosmole</td>
</tr>
<tr>
<td>MRP1</td>
<td>multi-drug resistance protein 1</td>
</tr>
<tr>
<td>NBC</td>
<td>sodium bicarbonate co-transporter (e.g., NBC1, NBC3, NBC4)</td>
</tr>
<tr>
<td>NHE</td>
<td>sodium hydrogen exchanger</td>
</tr>
<tr>
<td>NKCC1</td>
<td>sodium potassium chloride co-transporter isoform 1</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glucamine</td>
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<td>P</td>
<td>relative CO₂ permeability at the apical vs. basolateral membrane</td>
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<tr>
<td>pCMBS</td>
<td>p-chloromercuribenzenesulfonic acid</td>
</tr>
<tr>
<td>PEDF</td>
<td>pigment epithelium-derived factor</td>
</tr>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt;</td>
<td>intracellular pH</td>
</tr>
<tr>
<td>pro</td>
<td>propionate</td>
</tr>
<tr>
<td>PVR</td>
<td>proliferative vitreoretinopathy</td>
</tr>
<tr>
<td>pyr</td>
<td>pyruvate</td>
</tr>
<tr>
<td>R&lt;sub&gt;1&lt;/sub&gt;</td>
<td>apical lactate induced pH&lt;sub&gt;i&lt;/sub&gt;-response 1 (acidification)</td>
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<td>R&lt;sub&gt;2&lt;/sub&gt;</td>
<td>apical lactate induced pH&lt;sub&gt;i&lt;/sub&gt;-response 2 (alkalinization)</td>
</tr>
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CHAPTER 1: Introduction and Background

Section 1.1 – Overview

Our view of the external world begins at the photoreceptors, which capture light quanta and convert them into electrical signals that are transmitted to and interpreted in our brain as vision. This event involves a large amount of coordination between many different specialized cells within the retina, which continuously integrates, process, and transmit the visual signals to the brain (Yau, 1994; Lamb & Pugh, 2004; Stephen et al., 2008). However, photoreceptor function requires a large amount of energy, even more so than the brain. Energy (as ATP) is generated by mitochondria that are densely packed at the junction between photoreceptor inner and outer segments (Stone et al., 2008). As a result, metabolic waste-products (i.e., CO$_2$, lactic acid, and water) are released into the extracellular space that surrounds the photoreceptors – subretinal space (SRS). In the human eye, the SRS has a very small volume ($\approx$ 10 $\mu$L), thus even small accumulations of metabolic acids within the SRS can cause acidosis and increased osmotic pressure – both of which can be detrimental to photoreceptor function. It has been hypothesized that the retinal pigment epithelium (RPE), a specialized monolayer of cells adjacent to the photoreceptors (Fig. 1-1), prevents these potentially catastrophic conditions by actively transporting these photoreceptor-generated metabolic by-products from the SRS into the choroid. However, the mechanisms involved in this process have been unclear.

The goal of this thesis is to identify and understand the various mechanisms involved in CO$_2$, lactic acid, and water transport in human RPE. We used a primary cell culture model of fetal human RPE (Chapter 1), which has been shown to closely mimic
physiological and morphological characteristics of the native tissue (Lin et al., 1992; Quinn & Miller, 1992; la Cour et al., 1994; Quinn et al., 2001; Maminishkis et al., 2006; Adijanto et al., 2009; Wang et al., 2010). To study RPE pH regulatory mechanisms involved in CO₂ and lactic acid transport, intracellular pH (pHᵢ) in human RPE was measured by using a pHᵢ-sensitive fluorescence dye (Chapter 2). The transepithelial potential (TEP) was recorded with a pair of calomel electrodes linked to agar bridges placed in apical and basal baths (Chapter 2). Recording TEP is a highly sensitive method to detect acute (millisecond) changes in apical or basolateral membrane voltage (Vₐ & Vₖ) that reflects ion channel or transporter activity. In some experiments, we placed a reference microelectrode within the cell to record changes in Vₐ & Vₖ independently (Chapter 2). Combining these recording techniques with pharmacological interventions (see Table 2-4) and other maneuvers allows us to produce unequivocal physiological data on human RPE transporters and channels (Chapters 3, 4, & 5). Understanding the normal physiological role of mechanisms involved in metabolic acid transport provides a first critical step in understanding the pathophysiology of human ocular diseases. This analysis is required for the development of appropriate animal models suitable for preclinical experiments that can lead to phase I clinical trails and facilitate the development of therapeutic interventions and strategies for disease prevention.

This new work is described in three main chapters: (1) CO₂/HCO₃ transport in RPE (Chapter 3); (2) lactate transport in the absence of CO₂/HCO₃ (Chapter 4); (3) lactate transport and its interactions with HCO₃-transport mechanisms (Chapter 5). In Chapter 6, we present ideas for future projects and describe some supporting preliminary data.
Section 1.2 - Behind every successful retina is an intact retinal pigment epithelium

In 2002, 2.5% (160 million) of the world’s total human population was visually impaired, with 0.6% being completely blind (www.who.int/en/). The consequences of vision loss are severe: you lose a sense of yourself and the world around you. A reduced ability to independently perform essential daily functions diminishes an individual’s quality of life and imposes a burden on one’s family and the community. The RPE is involved in many vision-impairing diseases. For example, age-related macular degeneration (AMD) is the leading cause of blindness among the elderly (> 55 years) in the US and developed countries world-wide (Congdon et al., 2004).

Fig. 1-1: Anatomy of the eye: the neural retina and retinal pigment epithelium.
Light enters the eye through the cornea and passes through the lens and vitreous onto the retina, where it is absorbed by the visual pigment within the photoreceptors (rods and cones) (Fig. 1-1). The capture of a photon by the visual pigment activates visual phototransduction; a cascade of events that induces a rapid change in photoreceptor membrane potential (Yau & Hardie, 2009). This response is subsequently transmitted across the retinal layers and out of the retinal ganglion cells into the brain (Fig. 1-1). The rest of the retina serves to fine-tune and regulate the electrical input from the photoreceptors (bipolar, amacrine, and horizontal cells), and to protect and nourish the retinal cells (Müller cells) (Oyster, 1999). The retinal pigment epithelium (RPE) separates the photoreceptor from its major blood supply (choroid) and provides critical support to retinal photoreceptor function (Gallemore et al., 1998; Hughes et al., 1998; Strauss, 2005).

![Fig. 1-2: Schematic of retinal pigment epithelium cells depicting apical and basolateral membrane separation by tight junctions.](image)

Like other epithelia, the RPE is a polarized monolayer – its apical (facing photoreceptors) and basolateral (facing choroid) membranes are separated by tight and adherens junctions.
that link individual RPE cells to adjacent cells to form a confluent monolayer of cells (Fig. 1-2) (Nelson, 2003; Burke, 2008). The RPE apical and basolateral membranes are separated by tight junctions, which help establish cell polarity by acting as a “fence” that segregates the proteins that are trafficked to either the apical or basolateral membranes (Shin et al., 2006; Terry et al., 2010). RPE cell polarity is necessary for material (e.g., nutrients, metabolic by-products, growth factors) exchange and transport between photoreceptors, RPE, and choroid (Hughes et al., 1998; Rizzolo, 2007).

![Fig. 1-3: Anatomical relationship between retinal pigment epithelium and photoreceptor (Miller & Steinberg, 1977).](image)

To facilitate this process, the RPE apical membrane has numerous microvilli that ensheathes the outer segments of the photoreceptors (Fig. 1-3). This anatomical structure
increases the apical membrane surface area in contact with the SRS, thus facilitating RPE-photoreceptor interactions (e.g., material exchange and transport) due to the high surface/volume ratio. The RPE apical membrane and photoreceptors are not in physical contact; they are separated by a small volume ($\approx 10 \, \mu\text{L}$ in human) of extracellular space (subretinal space; SRS), whose ionic and pH homeostasis is maintained by active and passive transport of solute and fluid across the RPE. The RPE basolateral membrane faces the Bruch’s membrane and an extensive network of blood vessels (choriocapillaries) that form the choroidal blood supply (Fig. 1-4). The choroid has normally high blood flow rate; it accounts for $\approx 85\%$ of blood supply to the eye (Alm & Bill, 1973; Bill, 1975). As such, it constitutes the sole blood supply for retinal photoreceptors; the RPE transports nutrients, ions, fluid, and metabolic byproducts between photoreceptors and choroid (Hughes et al., 1998).

Fig. 1-4: The choroidal blood supply (Olver, 1990). (A) Choroidal vasculature viewed from the scleral side, showing arterioles (e.g., white arrow), venules (e.g., black arrow), and choriocapillaris (asterisk). (B) Choriocapillaris viewed from the retinal side.
Besides solute and fluid transport, the RPE also mediates important visual functions such as the retinal cycle (Rando, 1991; Lamb & Pugh, 2004), where retinal (vitamin A component of the visual pigment) is constantly cycled between the photoreceptors and the RPE to regenerate the visual pigment (rhodopsin) and maintain photoreceptor photoexcitability. In addition, the photoreceptors are highly susceptible to photodamage, thus accumulating toxic amounts of oxidized proteins and lipids within the photoreceptor discs, which are continuously shed and replaced at a high turnover rate (Nguyen-Legros & Hicks, 2000; Kevany & Palczewski, 2010). The RPE phagocytose shed photoreceptor discs and recycle its components back to the photoreceptor cilium – where new photoreceptor discs are synthesized. Failure of the RPE to phagocytose photoreceptor discs results in photoreceptor degeneration in rats (Vollrath et al., 2001) and in humans (Koenekoop, 2007). The RPE also secretes a wide variety of growth factors in a polarized manner to the neural retina (e.g., pigment epithelium-derived factor (PEDF)) and to the choroid (e.g., vascular endothelial growth factor (VEGF)); these growth factors help maintain the structural integrity of the neural retina and the choroidal vasculature (Ishida et al., 1997; Blaauwgeers et al., 1999; Jablonski et al., 2000; King & Suzuma, 2000; Schlingemann, 2004; Marneros et al., 2005). Loss of RPE function leads to photoreceptor degeneration and visual impairment; RPE damage leads to retina and choroid degeneration (Del Priore et al., 1995; Litchfield et al., 1997; Aramant & Seiler, 2004).
Section 1.3 – The retina and its high metabolism in light and in the dark

![Diagram of rod photoreceptor]

Fig. 1-5: Schematic of rod photoreceptor: The dark current circulates between the inner and outer segments.

Metabolic activity in the photoreceptors is among the highest of all human tissue (Wangsa-Wirawan & Linsenmeier, 2003); this energy is required to fuel phototransduction and to regenerate new photoreceptor discs (Kimble et al., 1980; Yu & Cringle, 2001). The photoreceptor is a polarized cell (Fig. 1-5); it expresses cGMP-gated cation (85% Na\(^+\) and 15% Ca\(^{2+}\)) channels at the outer segments and a K\(^+\)-selective channel and the electrogenic Na\(^+\)/K\(^+\) ATPase at the inner segment. The polarized
distribution of these channels allows for the circulation of a current around the inner and outer segments. This current (also called the dark current) is maximal in the dark; in light, phototransduction events lead to the depletion of cGMP at the outer segment, which causes the cGMP-gated cation channels to close (Yau & Hardie, 2009). The closure of these channels hyperpolarizes the photoreceptor membrane potential, which inhibits the release of glutamate neurotransmitter at the photoreceptor terminal (rod spherule or cone pedicle) into the synaptic cleft, where it activates 2\textsuperscript{nd} order neurons such as bipolar cells and horizontal cells (Oyster, 1999). These electrical signals are subsequently transmitted to the retinal ganglion cells and into the brain.

The dark current relies on the high Na\textsuperscript{+} gradient (inward) and a high K\textsuperscript{+} gradient (outward) across the photoreceptor plasma membrane. If these ionic gradients were not continuously sustained, the dark current eventually drives both Na\textsuperscript{+} and K\textsuperscript{+} gradients into equilibrium, and the photoreceptors would no longer respond to photoexcitation. The 3Na\textsuperscript{+}/2K\textsuperscript{+} ATPase at the inner segment maintains the Na\textsuperscript{+} and K\textsuperscript{+} gradients across the plasma membrane by using the energy of an ATP molecule to drive three Na\textsuperscript{+} ions out of the cell in exchange for two K\textsuperscript{+} ions. A recent study estimated that ATP consumption by rod photoreceptor increases \(\approx 4\)-fold in the dark (Okawa et al., 2008). In darkness, large amount of ATP is needed to maintain the dark-current, which requires a high 3Na\textsuperscript{+}/2K\textsuperscript{+} ATPase activity at the inner segments (Ames et al., 1992). In addition, ATP is used by Ca\textsuperscript{2+}-ATPase to recycle Ca\textsuperscript{2+} out of the cell (Okawa et al., 2008). Activity of these ATPase pumps increases [ATP], and increases [ADP], [inorganic phosphate], and [AMP], which in turn activates glycolysis, the TCA cycle, and oxidative phosphorylation.
Most of the ATP used by the photoreceptors is produced by oxidative phosphorylation in mitochondria located at the junction between the inner and outer segment of the photoreceptor (Fig. 1-5) (Stone et al., 2008). A recent study also provides evidence of mitochondria-independent oxidative phosphorylation at the photoreceptor disks (Panfoli et al., 2009). In the dark-adapted eye, photoreceptor O₂ consumption (oxidative metabolism) and energy production increases to meet the high ATP demand (Wangsa-Wirawan & Linsenmeier, 2003) – this depletes O₂ in the SRS. Consistent with this notion, in vivo O₂-sensitive microelectrode experiments on the cat eye show that SRS [O₂] levels is ≈ 0 mm Hg in the dark vs. ≈ 20 mm Hg in light (Wangsa-Wirawan & Linsenmeier, 2003). The depletion of local O₂ forces the photoreceptors to rely more on glycolysis (with lactate production) as a source of energy (Linsenmeier, 1986). Increased photoreceptor metabolism (aerobic and anaerobic) in the dark increases CO₂, lactic acid, and water release into the SRS. Metabolic acid accumulation in the SRS can cause acidosis that inhibits photoreceptor activity (Liebman et al., 1984) and is detrimental to the health of surrounding cells (i.e., Müller cells, photoreceptors, and RPE). In addition, pathological conditions that compromise the ability of RPE to clear fluid from the SRS can cause abnormal fluid accumulation, resulting in retinal detachment and photoreceptor death (Stone et al., 1999; Wickham et al., 2006; Nakazawa et al., 2007). The RPE protects the photoreceptors from these potentially destructive conditions in part by regulating pH- and ion- homeostasis of the SRS. Among its many functions, the RPE is responsible for the removal and delivery of metabolites to the choroidal blood supply.
Fig. 1-6: Blood supply of the inner retina (Fig. 6-25 from Oyster, 1999).

The neural retina has two blood supplies (Fig. 1-6) (Oyster, 1999): (1) the retinal blood circulation is located at the inner retina; (2) the choroidal blood supply is located distally behind the RPE. Early studies demonstrated that choroidal circulation is the major blood supply for the photoreceptors; blocking choroidal circulation results in photoreceptor degeneration without affecting other retinal cells (Oyster, 1999). Studies in porcine eye also show that most lactate released from the retina is transported to the choroid by the RPE (Wang et al., 1997b). The choroid is the main sink for the removal of most photoreceptor-generated metabolites: (1) blood flow rate in the choroid is significantly higher than in the retinal blood vessels (Alm & Bill, 1987); (2) metabolites released by the photoreceptor inner segments need to diffuse a shorter distance to the choroid (35 μm) than to the retinal capillary bed at the inner nuclear layer (70 μm) (Oyster, 1999).
Section 1.4 – Major ion and fluid transport mechanisms in RPE

Fig. 1-7: Morphology and functions of tight junctions (Sawada et al., 2003). (A) Schematic diagram of tight junction. (B) Tight junction strands on freeze-fracture replica. (C) The fence and barrier functions of tight junctions.

The RPE apical membrane is separated from the basolateral membrane by tight junctions (TJs) – TJs function as a fence that prevents intermixing of proteins and lipids between the apical and basolateral membrane domains (Fig. 1-7) (Rizzolo, 2007; Burke, 2008; Cereijido et al., 2008). In addition, TJs also form a selectively permeable barrier at the intercellular spaces between epithelial cells and its adjacent neighbors, thus limiting free diffusion of certain ions and large molecules across the paracellular pathway. The TJ is mainly composed of three families of transmembrane proteins: (1) occludin; (2) claudins;
(3) junctional adhesion molecules (JAMs). These TJ proteins then associate with several peripheral membrane scaffolding proteins (e.g., ZO-1), which in turn interacts with the actin-cytoskeleton (Shin et al., 2006). This interaction induces cytoskeleton assembly and reorganization within the cell and is critical for post-golggi protein delivery to its proper membrane domain (Etienne-Manneville & Hall, 2003; Nelson, 2003). These proteins allow the RPE to maintain a polarized distribution of ion channels, receptors, and transporters at its apical and basolateral membranes (Fig. 1-8) (Hughes et al., 1989; Lin & Miller, 1994; Kenyon et al., 1997; Hughes et al., 1998; Yang et al., 2008a; Adijanto et al., 2009).

**Fig. 1-8:** Ion channels and transporters in retinal pigment epithelium.

The RPE is one of the two epithelia in the body that expresses the 3Na/2K ATPase (a classical marker of epithelial polarity) at the apical membrane (Okami et al., 1990; Rizzolo, 1990; Gundersen et al., 1991; Hughes et al., 1998). The 3Na/2K ATPase is
essential for any transporting epithelium, because it maintains Na-gradient needed for vectorial (unidirectional) nutrient, ion, and fluid transport (Jaitovich & Bertorello, 2006). Besides the 3Na/2K ATPase, the RPE apical membrane contains a Na/H exchanger (NHE), an electrogenic Na/2HCO\textsubscript{3} co-transporter (NBC1), an electroneutral Na/HCO\textsubscript{3} co-transporter (NBC3), a Na/K/2Cl co-transporter (NKCC1), and an inwardly-rectifying K\textsuperscript{+}-channel (Kir 7.1). The basolateral membrane contains an electrogenic Na/nHCO\textsubscript{3} co-transporter (NBC; n ≥ 2), a Cl/HCO\textsubscript{3} exchanger (AE2), a Ba\textsuperscript{2+}-sensitive K\textsuperscript{+}-channel, Ca\textsuperscript{2+}-activated Cl-channels, and cAMP-activated cystic fibrosis transmembrane conductance regulator (CFTR). This segregated arrangement of proteins on separate membranes allows the RPE to coordinate solute transport vectorially from one side of the tissue to another. The major ions in most biological systems are Na\textsuperscript{+}, K\textsuperscript{+}, Cl\textsuperscript{-}, and HCO\textsubscript{3}-. [Na]\textsubscript{o} (∼ 140 mM) and [Cl]\textsubscript{o} (∼ 120 mM) levels are high in the extracellular space, but are low in RPE cytosol ([Na]\textsubscript{i} ∼ 15 mM and [Cl]\textsubscript{i} ∼ 70 mM respectively). In contrast, [K] is high in RPE cytosol (∼ 85 mM), but low in the extracellular space (2-5 mM). [HCO\textsubscript{3}-] is approximately equal inside and outside the cell (∼ 25 mM).

At the apical membrane, Cl is transported into the cell via the NKCC1, and Cl exits the basolateral membrane via Ca\textsuperscript{2+}-activated Cl-channels and CFTR. HCO\textsubscript{3} is transported across the apical membrane via NBC1 and NBC3 with the help of the strong Na-gradient. HCO\textsubscript{3} is transported out of the basolateral membrane via NBC and AE2 (a Cl/HCO\textsubscript{3} exchanger). Na enters the cell from the apical membrane via NKCC1 and NBC1. Although some of these Na is recycled out of the apical membrane via the 3Na/2K ATPase, the unrecycled Na leaves the basolateral membrane via the basolateral
membrane NBC. Na-channels or its activity has not been detected in the RPE, NBC is currently the only known Na-transport pathway at the basolateral membrane. K enters the cell from the apical membrane via NKCC1 and 3Na/2K ATPase, but most (if not all) K is recycled out of the apical membrane via Kir 7.1 K-channel. Other K-channels at the basolateral membrane provides $K^+$ as a counter-ion for $Cl^-$ and $HCO_3^-$ efflux from the RPE. Although ions are transported across the RPE plasma membrane via transporters and channels, some ions (i.e., $Na^+$ and $Cl^-$) can also diffuse across the tight junctions (see Fig. 1-8). In the paracellular pathway, Na is absorbed (from apical to basal) and Cl is secreted (from basal to apical). In the resting state, the RPE transports NaCl and NaHCO$_3$ from the apical side to the basal side to drive isoosmotic fluid transport across the epithelium. In addition to osmotically driven fluid transport, other mechanisms of fluid transport have also been described (Zeuthen, 2000).

Carbonic anhydrases (CAs) are zinc-metalloenzymes that catalyze the conversion between CO$_2$ and HCO$_3^-$ according to the following equation: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$. Several isozymes of CAs found in the RPE (CA II, CA IV, CA IX, and CA XIV) has been shown in other systems to functionally interact with HCO$_3^-$-transporters such as NBC1, NBC3, AE1, AE2, AE3 (Cl/HCO$_3^-$ exchangers) in a functional complex known as the bicarbonate-transport metabolon (Sterling et al., 2001; Alvarez et al., 2003; Loiselle et al., 2004; Nagelhus et al., 2005; Morgan et al., 2007; Casey et al., 2009; Svichar et al., 2009). In support of this metabolon theory, binding sites on AE1 and CA II for physical interactions with each other has been found (Vince et al., 2000; Vince & Reithmeier, 2000). Further, CA II is activated when it is physically bound to AE1.
(Scozzafava & Supuran, 2002). In RPE, several membrane-bound CAs (CA IV, CA XII, and CA XIV) are expressed exclusively at the apical surface (Wolfensberger et al., 1994; Nagelhus et al., 2005; Zhi et al., 2007), suggesting CA involvement in HCO₃⁻-transport at the apical membrane. Since the RPE functionally expresses several different HCO₃⁻-transport proteins (i.e., NBCs and AE2) at the apical and basolateral membranes, it is possible that their activities are dependent on CAs as illustrated in Fig. 1-9. Since HCO₃⁻-transport is linked to Na and Cl transport, the interactions between CAs and HCO₃⁻-transporters is indicative of the involvement of CAs in steady-state fluid transport across the RPE.

Fig. 1-9: Bicarbonate transport metabolon: HCO₃⁻-transporters (i.e., NBCs) interact with carbonic anhydrases (CAs) to maximize HCO₃⁻ transport across the RPE.
The close physiological relationship between HCO$_3$ and fluid-transport was demonstrated in frog RPE, where steady-state fluid-absorption was reduced by $\approx 70\%$ following HCO$_3$-removal from the bathing solutions (Hughes et al., 1984). Furthermore, the addition of a potent but non-specific CA-inhibitor, acetazolamide, has been shown to decrease steady-state fluid-transport in RPE. However, these $\textit{in vitro}$ results are in contrast to $\textit{in vivo}$ animal studies which suggest that acetazolamide (a non-specific CA-inhibitor) enhances retinal adhesion and SRS fluid clearance (Kita & Marmor, 1992; Wolfensberger et al., 2000). In addition, clinical studies showed that some (but not all) patients with macular edema respond to acetazolamide treatment by increasing SRS fluid clearance (Cox et al., 1988; Fishman et al., 1989). This difference in the effect of CA inhibition on fluid transport $\textit{in vivo}$ and $\textit{in vitro}$ will be discussed (in section 3.8), but the underlying mechanisms remain to be determined.

There are a number of retinal degenerative diseases (e.g., uveitis, retinitis pigmentosa, diabetic retinopathy, age-related macular degeneration) that lead to abnormal accumulation of fluid within the retina and subsequent loss of visual acuity (Wolfensberger, 1999). Consequently, there is significant interest in identifying mechanisms by which small molecules such as acetazolamide can be utilized to activate the fluid transport “engine” of the RPE to eliminate this fluid. Its efficacy in the clinic depends on the extent to which the underlying disease damages the RPE or its transport capability.
Section 1.5 – Lactate transport in RPE

Lactate is generally considered as a metabolic waste by-product, which accumulates in organs within our body to cause lactic acidosis and muscle fatigue (Hermansen, 1981). Research over the past three decades demonstrate that lactate is an intermediate metabolic substrate that undergoes oxidative metabolism in cells (Gladden, 2004; Philp et al., 2005; Kennedy & Dewhirst, 2010). The most well established example is the skeletal muscles, which contains type I oxidative fibers and type II glycolytic fibers. In this scheme, known as the lactate-shuttle model, type II fibers metabolize glucose into lactate upon exercise, which is directly taken up and oxidized by neighboring type I fibers (Peter et al., 1971; Baldwin et al., 1978). A similar lactate-shuttle mechanism was found between astrocytes and neurons in the brain, in which astrocytes generates lactate that is subsequently metabolized by the neighboring neurons to produce energy (Magistretti, 2006; Bergersen, 2007; Brown & Ransom, 2007; Pellerin et al., 2007; Pellerin, 2008), but this relationship between astrocyte and neuron is controversial (Chih et al., 2001; Gladden, 2004; Fillenz, 2005). In the retina, the lactate shuttling mechanism between Müller glia cells and photoreceptors has been described (Poitry-Yamate et al., 1995; Poitry et al., 2000): Müller cells metabolize glucose into lactate (Winkler et al., 2000), which is taken up by photoreceptors to be used as substrate for oxidative metabolism. However this theory of metabolic coupling between Müller cells and photoreceptors is also controversial (Winkler et al., 2004).

The retina is highly glycolytic. More than 80% of all glucose consumed by photoreceptors is converted to lactic acid (Wang et al., 1997a; Wang et al., 1997b;
Winkler et al., 2008) indicating that the retina is highly dependent on glycolysis as a source of ATP, even in the presence of oxygen (Winkler et al., 2000; Padnick-Silver & Linsenmeier, 2002; Winkler et al., 2004). Therefore, regardless of whether lactate derives from glutamate-induced lactate release from Müller glia cells, or as a product of photoreceptor aerobic glycolysis (or both), large amounts of lactic acid are generated and released from the retina into the SRS in light and in dark. This is consistent with the high lactate concentration (≈ 4 - 13 mM) in the SRS compared to that in blood (≈ 1 mM) (Adler & Southwick, 1992).

Lactic acid has a low pKa (3.9) and it exists predominantly in ionic form at physiological pH (7.4). Thus although lactic acid may diffuse passively across the plasma membrane, lactate is transported much more quickly via H⁺-coupled lactate transporters or anion-

![Diagram of lactate transport in retinal pigment epithelium](image-url)
Early physiological studies showed that RPE expresses a proton-coupled lactate transporter (1:1 $\text{H}^+:\text{Lac}^-$) at the apical membrane in various native and cultured RPE preparations (bovine, porcine, frog, and human; see Fig. 1-10) (Kenyon et al., 1994; la Cour et al., 1994; Lin et al., 1994; Zeuthen et al., 1996). This apical membrane H/Lac co-transporter in RPE was identified as MCT1, the first member in the family of monocarboxylate transporters (Philp et al., 2003b). Besides in RPE, MCT1 is also highly expressed in many other tissues in our body including skeletal muscle, heart, and brain (Bonen, 2001; Bonen et al., 2006; Chiry et al., 2006). At the RPE basolateral membrane, lactate is transported out of the cell via H/Lac co-transporters (MCT3 & MCT4) (Philp et al., 2001; Philp et al., 2003b) and a Cl/Lac anion exchanger (AE2) (Kenyon et al., 1994).

The importance of lactate transport in the mammalian eye was demonstrated in mice lacking MCT1, MCT3, and MCT4 expression – these mice gradually lose photoreceptor function and were completely blind within two months after birth (Hori et al., 2000; Philp et al., 2003a). Further, the affliction of MCT3-null mice with altered visual function reaffirmed the importance of lactate transport in the eye, more specifically in the RPE (Daniele et al., 2008).
CHAPTER 2: Materials and Methods

Section 2.1 – Human Fetal Retinal Pigment Epithelium (hfRPE) culture

Studying ion and fluid transport mechanisms of RPE using intact adult human RPE is highly impractical; adult human eyes are extremely difficult to obtain and are expensive if available (≈ $2000/pair). An alternative is to grow adult RPE cultures. However, culturing adult human RPE cells is complex and comes with many difficulties. For example, adult RPE cells are terminally differentiated and are non-mitotic, thus highly specialized culture media formulations supplemented with high levels of serum (10 – 20%) are needed to “coax” the RPE cells to proliferate (Valtink & Engelmann, 2009). In addition, culture conditions must also allow the RPE cultures to retain most of its in vivo characteristics. The development of RPE polarity and function in vivo are influenced by its interactions with the retina and the choroid (Rizzolo, 1991, 1999), which prompted the use of retinal or choroidal extracts in cell culture media to mimic in vivo conditions of the RPE (Hu & Bok, 2001; Engelmann & Valtink, 2004; Valtink & Engelmann, 2009). Nonetheless, typical RPE characteristics such as pigmentation and the cobblestone-like morphology are easily lost in cultures. Furthermore, RPE cell cultures have a tendency to dedifferentiate in a process called epithelial-mesenchymal transition into fibroblast-like cells with marked different morphological and physiological characteristics to that of RPE (MacDonald, 1994) (also see Chapter 6). There are also variations stemming from the production of retinal or choroid extracts and from the use of serum in the culture media. All these problems and complications results in large variations in the quality of human RPE cell cultures, which makes physiological studies with these cells difficult.
Early researchers observed that the success of human RPE culture decreases with increasing donor age (Engelmann & Valtink, 2004). Therefore, culturing RPE cells that are in the growth and development process should produce a more successful cell culture that is well-differentiated and retains \textit{in vivo} characteristics. Indeed, the best characterized models of human RPE today are human fetal RPE cultures developed by Bok and Miller laboratories (Hu & Bok, 2001; Maminishkis \textit{et al.}, 2006). Unlike Bok lab’s hfRPE culture, the hfRPE culture developed by the Miller lab does not require any retinal extracts in the culture media. In their formulation, every component of the media is commercially available and well-defined. These culture conditions allows for the “mass production” of highly differentiated and confluent hfRPE monolayers (Fig. 2-1) that possess many characteristics found in native RPE such as steady-state fluid absorption, ion transport, CO$_2$/HCO$_3$ transport, tight-junction formation, and growth factor secretion (Maminishkis \textit{et al.}, 2006; Li \textit{et al.}, 2007; Economopoulou \textit{et al.}, 2008;
Shi et al., 2008; Adijanto et al., 2009; Li et al., 2009). A major disadvantage of using native adult RPE (bovine or human) in physiological studies is the occlusion of the basolateral membrane by the thick choroidal vasculature, which significantly reduces the accessibility of drugs or solutions to the basolateral membrane. Using cultured hfRPE monolayers eliminates this problem; hfRPE cells are grown on a thin polyethylene membrane with numerous pores (0.4 μm diameter).
Section 2.2 – Fluorescence imaging experiments

Cultured hfRPE monolayer was incubated (at room temperature and 5% CO₂) in control Ringer solution containing 8 μM BCECF-AM (30 minutes) for pH₁-imaging, or 8 μM Calcein-AM (30 minutes) for volume measurements, or 10 μM (1 hour) Fura-2-AM for calcium-imaging (Invitrogen Corp., CA). Following dye-incubation, the hfRPE was incubated for another 30 minutes in control Ringer for at least 30 min before mounting onto a modified Üssing chamber (surface-area = 7.1 mm²). The Üssing chamber was mounted on the stage of a Zeiss axiovert-200 microscope equipped with a 20X plan-neofluar objective. The hfRPE was continuously perfused with Ringer solution (equilibrated with 5%/10%/85% CO₂/O₂/N₂ gas mixture at 34.5 °C) at a flow rate of 2 ml·min⁻¹. Excitation photons (440/480 nm-for BCECF; 480 nm for Calcein; 350/380 nm for Fura-2) were generated by a xenon light source and the specific wavelengths were selected with a monochromator, Polychrome IV (Photonics, CA). The emission fluorescence (535 nm) was collected, amplified and converted into electrical signals by a photomultiplier tube (Thorn, EMI).

pH₁-calibrations were performed by perfusing high-K calibration solutions (at pH = 6.8, 7.2, and 7.6) containing 20 μM nigericin into both solution baths. The average calibration parameters were used to linearly correlate fluorescence intensity to intracellular pH (pHᵢ) for all pH-imaging experiments. Fig. 2-2 is a schematic representation of the fluorescence imaging set-up. Cell volume measurement with calcein was not a ratiometric, therefore the data was fitted and normalized to an exponential curve (fluorescence signal = Ke⁻ᵇᵗ). Calibration was achieved by perfusing
control Ringer solutions (305 mOsm) with different osmolarities (i.e., 280 mOsm, 330 mOsm, and 355 mOsm) into both apical and basal baths. Fura-2 signals was calibrated by first perfusing both apical and basolateral membranes with Ca\textsuperscript{2+}-free Ringer solution (with 2 mM EGTA) before adding ionomycin (10 μM) into both solution baths. Next, control Ringer solution (1.8 mM Ca\textsuperscript{2+}) containing 10 μM ionomycin was added to both baths. After [Ca\textsuperscript{2+}]\textsubscript{i} reaches saturation, Ca\textsuperscript{2+}-free Ringer containing 5 mM MnCl\textsubscript{2} was added to quench Fura-2 fluorescence to reveal autofluorescence signals. For every experiment, autofluorescence levels were determined and subtracted from the data to obtain true Ca\textsuperscript{2+}-signals.

![Fig. 2-2: Schematic of the fluorescence imaging and electrophysiology set-up.](image-url)
Fluorescence dyes (i.e., BCECF, Fura-2, and calcein) are used in their ester-form (e.g., BCECF-AM), which are non-fluorescent but are cell permeable. The ester groups on BCECF-AM are cleaved by endogenous esterases within the cell, producing BCECF ions with multiple (4-5) negative charges. In its charged form, BCECF is membrane-impermeable. However, during the course of physiology experiments, fluorescence dye is lost through two main pathways: (1) photobleaching of the dye and (2) dye extrusion via an organic anion transporter (multi-drug resistant protein; MRP1 or ABCC1). Dye loss via photobleaching can be easily controlled by the following methods: (1) using a neutral-density filter (reduce light intensity); (2) controlling xenon-lamp intensity; (3) reducing exposure time and interval (20 ms exposure every 1.5 seconds). On the other hand, the control of dye extrusion via ABCC1 is more difficult and is the major cause of dye-loss in imaging experiments with hfRPE cells. This can be demonstrated by a simple test where excitation fluorescence was stopped for a long period of time (10 minutes) – fluorescence emission was reduced at the same rate with or without fluorescence excitation.

Fluorescence dye extrusion by ABCC1 is a function of two factors: (1) Ringer solution temperature (the higher the temperature, the faster the dye-leakage); (2) RPE viability (unhealthy or stressed RPE has a faster dye-leakage). Dye-loss is extremely fast at 37 °C. However, a low temperature reduces the activities of many proteins in the RPE – 34 °C strikes a good balance. When RPE cells experience prolonged periods of stress (e.g., alkalosis, acidosis, Na-free conditions), fluorescence dye-leakage was significantly faster. From these observations, it is tempting to speculate that either ABCC1 expression or
activity is upregulated by cell stress – alleviating cell stress should reduce dye-leakage. In support of this possibility, earlier pH$_i$-imaging experiments use Ringer solution supplemented with glutathione or glutaMAX™ (antioxidant) to help reduce dye-leakage. A more direct but effective method to prevent dye-leakage is the addition of probenecid, an ABCC1 inhibitor (Feller et al., 1995; de Jong et al., 2003), to all Ringer solution used in all imaging-experiments (Kenyon et al., 1997). However, high concentrations of probenecid show slight inhibition of Cl/HCO$_3$ exchange (Zeidel et al., 1986). Probenecid also inhibits SMCT (Coady et al., 2004), a Na-linked lactate co-transporter expressed in RPE (Martin et al., 2007). Therefore, a low concentration of probenecid (0.5 mM) is added to all Ringer solutions used in imaging experiments.
The intrinsic buffering capacity ($\beta_i$ mM/pH units) of the hfRPE cells was determined by using a previously described method (Weintraub & Machen, 1989) and was fitted to a third-order polynomial: 

$$
\beta_i = -93.4 \, pHi^3 + 2150.4 \, pHi^2 + 16483.6 \, pHi + 42065.6 \quad \text{for} \quad pHi < 7.35,
$$

and for $7.35 \leq pHi \leq 7.7$, $\beta_i = 9.06$. The total buffering capacity ($\beta_{total}$) was then calculated with the equation, 

$$
\beta_{total} = \beta_i + \beta_{HCO_3} = \beta_i + 2.3[HCO_3^-]. \quad \text{[HCO}_3^-] \text{ was estimated from the Henderson-Hasselbalch equation with the assumption that intracellular CO}_2 \text{ level is 5%}. 
$$

$H^+$-flux was determined by multiplying $\beta_{total}$ by an estimate of the initial $dpHi/dt$ determined from the pH$_i$ response.
Section 2.4 – Transepithelial potential and total tissue resistance

As described earlier, the RPE maintains a polarized distribution of different channels and transporters at its apical and basolateral membranes, which are separated by the tight junction. This results in different apical and basolateral membrane voltages ($V_A$ and $V_B$), which gives rise to the transepithelial potential (TEP) of the RPE: $\text{TEP} = V_B - V_A$. Since $V_A$ and $V_B$ are directly affected by electrogenic (carrying net ionic charge) transport processes, the measurement of TEP provides a sensitive method to study ion channels and electrogenic transporters at the apical and basolateral membranes of the RPE. Early studies of epithelial electrophysiology showed that the electrical properties of the RPE can be simplified and analyzed in the form of an electrical circuit model as shown in Fig. 2-4 (Hughes et al., 1998).

Fig. 2-4: Equivalent circuit of the retinal pigment epithelium.
From the circuit analysis, \( V_A = E_A - I_S R_A \) and \( V_B = E_B + I_S R_B \). \( E_A \) and \( E_B \) are the “batteries” of the apical membrane and basolateral membranes respectively. \( E_A \) is the combination of all potentials that derives from the ion concentration gradients and their respective ion channel conductivity at the apical membrane: 

\[
E_A = \frac{g_K E_K + g_{NBC1} E_{NBC1}}{g_K + g_{NBC1}}. 
\]

\( g_K \) and \( g_{NBC1} \) are conductances of electrogenic processes (Kir7.1 and NBC1) at the apical membrane (conductance is the reciprocal of resistance). Similarly,

\[
E_B = \frac{g_K E_K + g_{NBC1} E_{NBC1} + g_{Cl} E_{Cl}}{g_K + g_{NBC1} + g_{Cl}}. 
\]

Since \( E_A \) and \( E_B \) have non-equivalent values, a current (\( I_S \), shunt current) circulates around the epithelium. The shunt current,

\[
I_S = \frac{E_A - E_B}{R_A + R_B + R_S}, 
\]

is carried mainly by \( \text{Na}^+ \) and \( \text{Cl}^- \) ions. \( R_A \) and \( R_B \) are apical and basolateral membrane resistances: 

\[
R_A = \frac{1}{g_A} = \frac{1}{g_K + g_{NBC1}}; \quad R_B = \frac{1}{g_B} = \frac{1}{g_K + g_{NBC1} + g_{Cl}}. 
\]

\( I_S \) depolarizes (more positive) the apical and hyperpolarizes (more negative) the basolateral membrane. Conventionally, the shunt current has a negative value.

Experimentally, TEP is measured with a pair of calomel electrodes in series with Ringer solution bridges (3 %wt/v agar) placed in the apical and basal baths of the Üssing chamber (Fig. 2-2).
Fig. 2-5: Measuring total tissue resistance ($R_T$).

The total tissue resistance ($R_T$) is experimentally determined by passing a 2 µA Current across the hfRPE monolayer (once every 45 seconds) with Ag/AgCl electrodes placed at the apical and basal baths and measuring the voltage drop (i.e., $\Delta TEP$) across the tissue.

$R_T$ is then calculated with Ohm’s law: $R_T = \frac{\Delta TEP \cdot Area}{Current}$. Area is the cross-sectional surface area of the RPE that is exposed to the apical or basolateral baths. As shown in Fig. 2-5, $R_T$ is a combination of apical and basolateral membrane resistances ($R_A$ and $R_B$), and tight junction resistance ($R_S$; shunt resistance).
Section 2.5 – Intracellular microelectrode recordings

Although TEP recording allows for the detection of changes in $V_A$ and $V_B$, a TEP response can have two different interpretations. For example, an increase in TEP can be interpreted as: (1) an increase in $V_B$, or (2) a decrease in $V_A$. By placing a microelectrode reference within the cell, $V_A$ and $V_B$ can be measured separately (Fig. 2-6). This method provides valuable information on ion-transport mechanism at the apical or basolateral membrane.

Fig. 2-6: Measuring apical and basolateral membrane voltages ($V_A$ & $V_B$) with intracellular microelectrodes.

In addition to measuring $V_A$ and $V_B$, when a current is passed across the tissue in the presence of an intracellular microelectrode, the ratio of $R_A/R_B$ can be determined by measuring the current induced deflections in $V_A$ and $V_B$. 
As shown in Fig. 2-7, the ratio of the current induced deflections in $V_A$ and $V_B$ (equations 1 and 2) is: 

\[
\frac{\Delta V_A}{\Delta V_B} = -\frac{R_A}{R_B}.
\]
Section 2.6 – Steady-state fluid-transport measurement

The polarized distribution of the various ion-transport mechanisms at the apical and basolateral membranes allow for vectorial transport of solute (Na, Cl, and HCO₃⁻) and water from the apical side to the basal side. By measuring the capacitance between the probe and the basal bath (electrically grounded), we can monitor the steady increase in fluid level in the basal bath, which is directly proportional to the steady-state fluid transport rate of the RPE (Fig. 2-8). In this set-up, hfRPE monolayers were mounted in a modified Üssing chamber and the rate of transepithelial water flow ($J_V; \mu l \cdot cm^{-2} \cdot hr^{-1}$) was measured using a capacitance probe at the basal bath of the chamber (Hughes et al., 1984). In addition, the TEP (mV) and $R_T (\Omega \cdot cm^2)$ of the hfRPE monolayer were simultaneously measured by injecting a known current (5 μA) via Ag/AgCl electrodes that were connected to the solution baths with Ringer solution bridges (4 %wt agar).

Fluid transport experiments were performed in a Steri-Cult™ CO₂ incubator (Thermo Electron Corp; OH, Marietta) set at 5%/10%/85% CO₂/O₂/N₂ and 37 °C.

Fig. 2-8: Schematic of the fluid-transport measurement set-up.
Section 2.7 – Ringer solution composition, materials, and methods

Physiology experiments are performed with physiological saline solution (Ringer solution). Two main types of Ringer solution are used: (1) CO$_2$/HCO$_3$ buffered; (2) HEPES buffered. CO$_2$/HCO$_3$ buffered Ringer contains 26.2 mM of HCO$_3$ and requires continuous bubbling of 5% CO$_2$ (in a gas mixture containing 5%/10%/85% CO$_2$/O$_2$/N$_2$) to maintain a pH of 7.5 at 37 °C. On the other hand, HEPES buffered Ringer do not contain CO$_2$ or HCO$_3$, and is titrated to pH 7.5 (at 37 °C) with NMDG (base). CO$_2$/HCO$_3$ buffered Ringer is more physiologically relevant as CO$_2$/HCO$_3$ is the major buffer system used in our body to control cellular pH. In some cases CO$_2$/HCO$_3$-free Ringer (HEPES buffered) is very useful: (1) simplicity: HEPES buffered solutions do not require constant bubbling or incubation with 5% CO$_2$; (2) to study HCO$_3$-transport: CO$_2$/HCO$_3$-free condition eliminates HCO$_3$ transport activity; (3) removing the major intracellular buffering system in the cell: acid-coupled transport mechanisms (e.g., H$^+$/Lac co-transporter) can be easily studied.

For imaging and electrophysiology experiments involving hfRPE cultures, the Ringer solutions (Table 2-1) closely mimics the ionic composition and osmolarity of the cell culture media (Minimum Essential Medium Eagle; MEM), which has a final osmolarity of 310 mOsm (after addition of 5% serum, amino-acid supplements, antibiotics, and taurine). Control Ringer solution contains (in mM): 142.7 Na$^+$, 126.1 Cl$^-$, 26.2 HCO$_3^-$, 5 K$^+$, 0.5 Mg$^{2+}$, 1.8 Ca$^{2+}$, 2 Taurine, 5 Glucose. The solution is equilibrated with 5%/10%/85% CO$_2$/O$_2$/N$_2$. Sucrose was added to the Ringer to reach osmolarity of 305 mOsm. Low Cl (1 mM) Ringer (pH 7.5 when equilibrated with 5% CO$_2$) was prepared
by replacing all Cl-salts with gluconate-salts (except for MgCl₂). The high gluconate concentration (128.7 mM) in the low Cl Ringer requires consideration of the strong Ca²⁺ and Mg²⁺ chelating ability of gluconate. By using the stability constants of Ca-gluconate (16.22 L·mol⁻¹) and Mg-gluconate (5.01 L·mol⁻¹) (Furia, 1972; Abercrombie et al., 1983), the estimated free [Ca²⁺] and [Mg²⁺] in the low Cl Ringer are 0.6 and 0.3 mM, respectively. Therefore, the low Cl Ringer was supplemented with additional Ca²⁺ (to 5.7 mM) and Mg²⁺ (to 0.84 mM) to give free [Ca²⁺] and [Mg²⁺] of 1.8 mM and 0.5 mM, respectively. Low HCO₃ Ringer (2.62 mM HCO₃; pH 6.5 when equilibrated with 5% CO₂) was prepared by replacing 23.58 mM NaHCO₃ with equimolar Na-gluconate. Ca²⁺/Mg²⁺-free Ringer was made by replacing all CaCl₂ and MgCl₂ with 4.6 mM NMDG-Cl. Na-free Ringer was made by replacing all Na-salts with NMDG. NMDG-Cl was prepared by titrating NMDG solution with HCl. NMDG-HCO₃ was prepared by equilibrating NMDG solution with 5%/10%/85% CO₂/O₂/N₂ gas mixture.

Table 2-1. Ringer solutions for CO₂/HCO₃ transport experiments (all values in mM).

<table>
<thead>
<tr>
<th></th>
<th>Control Ringer</th>
<th>Low Cl Ringer</th>
<th>Low HCO₃ Ringer</th>
<th>Ca²⁺ &amp; Mg²⁺ free Ringer</th>
<th>Na-free Ringer</th>
</tr>
</thead>
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<td>116.5</td>
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<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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</tr>
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</tr>
<tr>
<td>CaCl₂</td>
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<tr>
<td>MgCl₂</td>
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<tr>
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<td>Ca-gluconate</td>
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<td>Mg-gluconate</td>
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<td>NMDG-HCO₃</td>
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<td></td>
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</table>

All Ringer solutions have pH 7.4 after equilibration with 5%/10%/85% CO₂/O₂/N₂ gas mixture.
In all lactate transport experiments, CO\textsubscript{2}/HCO\textsubscript{3}-buffered Ringer (pH 7.4 with 5\% CO\textsubscript{2}) has the following ionic composition (Table 2-2) (in mM): 133.7 Na\textsuperscript{+}, 116.1 Cl\textsuperscript{-}, 26.2 HCO\textsubscript{3}\textsuperscript{-}, 5 K\textsuperscript{+}, 0.5 Mg\textsuperscript{2+}, 1.8 Ca\textsuperscript{2+}, 2 Taurine, 5 Glucose. Lactate Ringer was prepared by adding 20 mM Na-lactate, and replacing 20 mM of NaCl with NMDG-Cl.

Table 2-2.  Ringer solutions for lactate transport experiments (all values in mM).

<table>
<thead>
<tr>
<th></th>
<th>Control Ringer</th>
<th>Control Ringer w/ lactate</th>
<th>Na-free Ringer</th>
<th>Na-free Ringer w/ lactate</th>
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<tr>
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<td>106.5</td>
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</tr>
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<td>Glucose</td>
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<td>NMDG-Lactate</td>
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</table>

All Ringer solutions have pH 7.4 after equilibration with 5\%/10\%/85\% CO\textsubscript{2}/O\textsubscript{2}/N\textsubscript{2} gas mixture.

The CO\textsubscript{2}/HCO\textsubscript{3}-free Ringer (HEPES buffered; Table 2-3) has the same ionic composition as CO\textsubscript{2}/HCO\textsubscript{3} Ringer except: (1) all HCO\textsubscript{3} was replaced with gluconate; (2) 7 mM HEPES acid was added to the CO\textsubscript{2}/HCO\textsubscript{3}-free Ringer and titrated with NMDG to reach pH 7.4 (at 36.9 °C). Cl-free Ringer solution contains CaSO\textsubscript{4} instead of CaCl\textsubscript{2}; mercury sulfate electrodes (sat. K\textsubscript{2}SO\textsubscript{4}; Koslow, NJ) were used instead of calomel electrodes. Since Cl-free Ringer was made by replacing all Cl with gluconate (138.7 mM), 5.9 mM CaSO\textsubscript{4} and 0.85 mM Mg-gluconate was added to this Ringer to compensate for Ca\textsuperscript{2+} and Mg\textsuperscript{2+} chelation by gluconate. Na-free Ringer containing lactate was prepared by adding NMDG-lactate, which was made by titrating NMDG solution with lactic acid.
Table 2-3. CO$_2$/HCO$_3$-free Ringer Solutions for lactate transport experiments (all values in mM).

<table>
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<tr>
<th></th>
<th>CO$_2$/HCO$_3$ free Ringer</th>
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<th>Cl &amp; CO$_2$/HCO$_3$ free Ringer w/ lactate</th>
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<td></td>
</tr>
</tbody>
</table>

All Ringer solutions should have pH 7.4 ± 0.1, add more or less NMDG to obtain pH 7.4.

Dorzolamide hydrochloride was purchased from U.S. Pharmacopeia (MD, Rockville). DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) and Nigericin were purchased from Calbiochem (CA, La Jolla). pCMBS (p-chloromercuribenzenesulfonic acid) was purchased from Toronto Research Chemicals (Ontario, Canada). Benzolamide was a kind gift from Dr. Erik Swenson of the University of Washington (WA, Seattle). All other chemicals were purchased from Sigma-Aldrich Co. (MO, St Louis).

In all physiological experiments, intracellular pH, TEP, and R$_T$ were recorded simultaneously. The hfRPE produces a response (i.e., pH$_i$, TEP, or R$_T$ changes) when Ringer solution with a special composition (e.g., low HCO$_3$ Ringer, or 20 mM lactate Ringer) was perfused into the apical or basal baths. For every experiment, two initial
control responses (2 min per pulse) were obtained to assure consistency and reversibility of the control responses. Next, the hRPE was exposed to drug/condition for 4-5 minutes (or until the resting pH and TEP stabilizes) before another response was obtained (in the continued presence of the drug/condition). After washing out the drug or returning to control condition, a control response was obtained to assess the reversibility of the drug/condition’s effect on the transporter activity corresponding to the control pulses.

Table 2-4. List of compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (μM)</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetazolamide</td>
<td>100</td>
<td>Membrane permeable CA inhibitor (non-specific)</td>
</tr>
<tr>
<td>Amiloride</td>
<td>1000</td>
<td>Inhibits Na⁺/H⁺ exchanger</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>2000</td>
<td>Inhibits K⁺-channels</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>20</td>
<td>Membrane permeable Ca²⁺ chelator</td>
</tr>
<tr>
<td>Benzolamide</td>
<td>10</td>
<td>Membrane permeable CA inhibitor (non-specific)</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>100</td>
<td>Inhibits Na⁺/K⁺/2Cl⁻ co-transporter</td>
</tr>
<tr>
<td>CFTRinh-172</td>
<td>10</td>
<td>Inhibits CFTR Cl-channel</td>
</tr>
<tr>
<td>DIDS</td>
<td>500</td>
<td>Inhibits anion channels and transporters</td>
</tr>
<tr>
<td>Dorzolamide</td>
<td>250</td>
<td>Membrane permeable CA inhibitor (non-specific)</td>
</tr>
<tr>
<td>Ethoxzolamide</td>
<td>10</td>
<td>Membrane permeable CA inhibitor (non-specific)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>40</td>
<td>Elevates cAMP to activate CFTR Cl-channel</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>10</td>
<td>Ca²⁺-ionophore for [Ca²⁺], calibration</td>
</tr>
<tr>
<td>Niflumic acid</td>
<td>100</td>
<td>Inhibits MCT1 and Ca²⁺-dependent Cl-channels</td>
</tr>
<tr>
<td>Nigercin</td>
<td>20</td>
<td>H⁺/K⁺ exchanger (ionophore) for pH, calibration</td>
</tr>
<tr>
<td>Ouabain</td>
<td>100</td>
<td>Inhibits 3Na⁺/2K⁺ ATPase</td>
</tr>
<tr>
<td>pCMBS</td>
<td>50</td>
<td>Inhibits MCT1</td>
</tr>
<tr>
<td>probenecid</td>
<td>500</td>
<td>Inhibits organic anion transporter</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>200</td>
<td>Inhibits CIC-2 Cl-channel and Na⁺/H⁺ exchanger</td>
</tr>
</tbody>
</table>
CHAPTER 3: CO$_2$-induced Ion and Fluid Transport in RPE

Section 3.1 – Introduction

The retinal pigment epithelium (RPE) is a polarized monolayer of cells that is part of the blood-retina barrier in the back of the vertebrate eye. This epithelium separates the choroidal blood supply from the extracellular or subretinal space (SRS) that surrounds the retinal photoreceptors. By transporting ions and fluid from the SRS to the choroid (Hughes et al., 1998; Maminishkis et al., 2002), the RPE plays a critical role in maintaining the volume and chemical composition of the SRS. Large amounts of CO$_2$ and H$_2$O are deposited into the SRS due to the high metabolic activity of the photoreceptors (Wangsa-Wirawan & Linsenmeier, 2003). The choroid’s high blood circulation, $\approx$ 1200 mL/min/100 g (Alm & Bill, 1987) and its proximity to the photoreceptor inner segments makes the choroid a very effective sink for the removal of these metabolites. Failure of the RPE to remove CO$_2$ would result in SRS acidosis detrimental to retinal function (Sillman et al., 1972; Meyertholen et al., 1986; Takahashi et al., 1993). In addition, abnormal accumulation of fluid in the SRS can cause retinal detachment and degeneration (Fisher et al., 2005).

*In vivo* studies of retinal metabolism show that SRS CO$_2$ level increases following the transition from light to dark (Wangsa-Wirawan & Linsenmeier, 2003). Since CO$_2$ is normally highly membrane permeable, the delivery of CO$_2$ from the SRS into the choroid was assumed to be achieved via passive diffusion. We show that the higher apical to basolateral membrane surface area results in a 10-fold higher CO$_2$ diffusion rate across the apical membrane than across the basolateral membrane. The relatively lower CO$_2$-
permeability at the basolateral membrane allows it to act as a bottleneck for CO₂ diffusion, thus trapping CO₂ within the cell. This accumulation of CO₂ in the cell causes the subsequent hydration of CO₂ into HCO₃⁻ by the catalytic activity of CA II. This in turn stimulates HCO₃⁻-efflux at the basolateral membrane. Therefore, the increase in SRS CO₂ level increases net NaHCO₃ absorption, which in turn drives solute-linked fluid transport across the RPE. In the transition from light to dark, the RPE can respond to the increased metabolic load by increasing the clearance of CO₂/HCO₃⁻ and fluid from the SRS. This would help protect and maintain the health and integrity of the retina/RPE complex by preventing acidosis in the subretinal space and an abnormal separation of retina and RPE.
Section 3.2 – CO₂ permeability at the apical and basolateral membranes

The study began with the unusual observation that in hfRPE, perfusing 13% or 1% CO₂ equilibrated Ringer to the apical membrane produced significantly larger pHᵢ responses than at the basolateral membrane (Fig. 3-1).

Fig. 3-1: 13% Apical or basal CO₂ induced pHᵢ, TEP, and RT responses.

Fig. 3-1 shows that increasing CO₂ from 5% to 13% in the apical or basal baths acidified the hfRPE by ≈ 0.25 and ≈ 0.04, respectively. Data from 13 experiments shows that 13% apical CO₂ decreased pHᵢ by 0.23 ± 0.03, from 7.37 ± 0.05 to 7.14 ± 0.06; in contrast, the 13% basal bath CO₂ induced acidification (ΔpHᵢ = 0.03 ± 0.01) was almost 8-fold smaller. Similarly in Fig. 3-2, decreasing CO₂ from 5% to 1% in the apical or basal baths alkalinized the hfRPE by ≈ 0.35 and ≈ 0.03, respectively.
In four experiments, decreasing apical or basal bath CO\textsubscript{2} from 5\% to 1\% alkalinized the cell by 0.41 ± 0.05 and 0.03 ± 0.03, respectively. The CO\textsubscript{2}-induced changes in TEP and R\textsubscript{T} were relatively small and not statistically significant. In freshly isolated native hfRPE preparations, 13\% apical CO\textsubscript{2} also caused significantly larger acidification ($\Delta p\text{H}_{i} = 0.29 ± 0.04$) than 13\% basal CO\textsubscript{2} ($\Delta p\text{H}_{i} = 0.03 ± 0.02$; n = 4; p < 0.05). This difference in the apical/basolateral CO\textsubscript{2}-induced pH\textsubscript{i} response is even more pronounced in bovine RPE-choroid preparations: no pH\textsubscript{i} response to 13\% basal CO\textsubscript{2} was observed, but a significant acidification was produced by 13\% apical CO\textsubscript{2} ($\Delta p\text{H}_{i} = 0.39 ± 0.09$; n = 6). In addition, significant differences between the 13\% apical and basal bath induced pH\textsubscript{i} responses were also observed in native fetal human RPE-choroid preparations.
Apical membrane processes increase the effective apical surface area of native frog RPE-
choroid by $\approx 30$-fold relative to the basolateral surface area (Miller & Steinberg, 1977).
Electron micrographs of hfRPE provide evidence for similar structures in hfRPE
(Maminishkis et al., 2006) and supports the notion of a relatively larger apical surface
area. This difference suggests a possible basis for the $\approx 8$-fold difference in the $\Delta pH_i$
produced by altering CO$_2$ (from 5 to 13%) in the apical versus basal bath. We can also
determine the relative CO$_2$-permeability of the apical vs. the basolateral membrane by
using the total buffering capacity of the cell (sections 2.3 & 3.11). With this method, we
calculated that the RPE apical membrane has a 10-fold higher CO$_2$ permeability than the
basolateral membrane.

hfRPE monolayers grown on transwell filters lack Bruch’s membrane and a thick
choroidal vasculature – it is flimsy and is easily damaged/stretched by the pressures
exerted by the constant apical and basal perfusion of Ringer solution. Therefore, a mesh
(250 $\mu$m thick) is placed under the basolateral surface of the hfRPE monolayer for
structural support (Fig. 3-3). However, CO$_2$-diffusion into the cell from the basal bath
may be hindered by the mesh and by the transwell filter itself. To eliminate the mesh as a
possible diffusion barrier, we show that the 13% basal bath CO$_2$ produced the same $\Delta pH_i$
with or without the mesh. To test if the transwell filter is a diffusion barrier, the hfRPE
monolayer was uniformly damaged by mounting its apical surface facing the mesh (Fig.
3-3).
Fig. 3-3: RPE monolayer damage by plastic mesh. Upper panel – intact RPE monolayer with proper mesh position. Lower panel – RPE monolayers are damaged by mounting the mesh on top of the RPE apical membrane.

This configuration allows CO\(_2\) from the basal bath to diffuse through the intact filter to reach the apical membrane surface by diffusing through the empty spaces (where the RPE were damaged). If the filter was a significant barrier to CO\(_2\), the difference between 13% apical and basal CO\(_2\)-induced \(\Delta\)pH\(_i\) in the damaged hfRPE would be similar to that in an intact hfRPE (≈ 8-fold).
However as shown in Fig. 3-4, 13% basal CO$_2$ caused a significantly larger acidification in the damaged hfRPE monolayer compared to intact hfRPE (compare with Fig. 3-1). In five damaged RPE monolayers tested, the difference in CO$_2$-induced $\Delta$pH$_i$ was $\approx$ 2.5-fold and the calculated relative CO$_2$ permeability was $3.0 \pm 1.5$. This indicates that the filter did limit CO$_2$-diffusion rate, but that alone cannot account for the large difference in relative difference in CO$_2$-permeability of the apical and basolateral membranes of intact hfRPE monolayers. From this observation, we infer that the basolateral membrane is relatively less permeable to CO$_2$ than the apical membrane.
There are two interpretations for this observed difference in CO$_2$ permeability: (1) apical CO$_2$-permeability is higher because of its relatively larger total surface area; (2) the chemical and physical composition of basolateral membrane is a CO$_2$-barrier that completely blocks CO$_2$ diffusion, similar to the apical membranes of gastric or colonic epithelia (Waisbren et al., 1994; Endeward & Gros, 2005). We can eliminate the latter possibility with the following experiment: the basal bath CO$_2$ was increased from 5 to 13% when we stop apical perfusion (Fig. 3-5). Stopping apical perfusion eliminates the convective flow at the apical bath, thus increasing the thickness of the unstirred layer at the apical membrane surface and reduces CO$_2$ diffusion out of the apical membrane. If the basolateral membrane does maintain a CO$_2$-barrier, then reducing the rate of CO$_2$ diffusion would not affect the 13% basal CO$_2$ induced pH$_i$-response.

![Fig. 3-5: 13% basal CO$_2$ induced pH$_i$, TEP, and R$_T$ responses in the absence apical perfusion.](image-url)
However when apical perfusion was stopped, the 13% basal CO$_2$-induced $\Delta$pHi was $\approx$ 3-fold greater than with continuous perfusion ($p < 0.05; n = 4$). This observation indicates that more of the CO$_2$ that diffuses into the basolateral membrane accumulates within the cytosol to cause the larger acidification mainly due to a reduced CO$_2$-efflux from the apical membrane. From this result, we infer that the basolateral membrane is CO$_2$ permeable and that the relatively higher apical membrane CO$_2$ permeability is probably due to its larger surface area.

The tight junction prevents the free flow of ions, large molecules (e.g., proteins or glucose), and water across the epithelium. Since CO$_2$ is a gas and is a small molecule, it may readily cross the tight junctions. If true, CO$_2$ may diffuse from the basal bath, across the tight junction into the apical bath, where it enters the cell from the apical membrane. To test this possibility, tight junctions were disrupted by removing all Ca$^{2+}$ and Mg$^{2+}$ from both solution baths for 15 minutes before adding 13% CO$_2$ Ringer to the basal bath (Fig. 3-6). This maneuver disrupts tight junctions by dissociating the link between tight junction proteins (claudins and occludin) and the intracellular adaptor proteins (e.g., ZO-1, -2, -3) (Brown & Davis, 2002; Rothen-Rutishauser et al., 2002). Since $R_T \approx$ tight junction resistance ($R_S$) in cultured hfRPE, the observation that [Ca$^{2+}$]$_o$ and [Mg$^{2+}$]$_o$ removal decreases $R_T$ (-17 ± 7 $\Omega \cdot$cm$^2 \cdot$min$^{-1}; n = 5$) indicates disruption of tight junctions. When control Ringer was returned to both solution baths, $R_T$ slowly recovered at a rate of 12 ± 4 $\Omega \cdot$cm$^2 \cdot$min$^{-1}$. However, the 13% basal CO$_2$-induced acidification was identical to control even in the absence of extracellular Ca$^{2+}$ ($p > 0.05; n = 5$), suggesting that the basolateral membrane is the main pathway for CO$_2$-entry from the basal bath.
Another possible CO₂-transport mechanism arises from the ability of aquaporin 1 (AQP1) to function as a CO₂ channel (Cooper & Boron, 1998; Endeward et al., 2006). In cultured hfRPE cells, AQP1 mRNA is highly expressed in human RPE (Wang and Miller, ARVO 2007, #6034). In addition, AQP1 was also detected specifically at the apical membrane (data not shown), corroborating an earlier study on rat RPE (Stamer et al., 2003). However, pCMBS (1 mM; non-specific AQP1 inhibitor) did not block or inhibit 13% apical CO₂ induced acidification (n = 3), inconsistent with the AQP1 hypothesis.

Fig. 3-6: 13% basal CO₂ induced pHᵢ, TEP, and Rᵣ responses in the absence of Ca²⁺ and Mg²⁺ in both apical and basal baths.
This asymmetry in apical and basolateral membrane CO₂-permeability has a very unique function. This feature allows the RPE to “trap” CO₂ within the cell. Accumulation of CO₂ in the cytosol favors CO₂ conversion into HCO₃⁻ by the catalytic activity of cytosolic carbonic anhydrase II (CA II). CA II-mediated formation of HCO₃⁻ in the cell stimulates HCO₃⁻-efflux from the basolateral membrane via HCO₃⁻-transporters. However, besides simple diffusion, CO₂ can also be transported across the apical membrane in its hydrated form, HCO₃⁻, via transporters. In this regard, there is strong electrophysiological evidence for an electrogenic Na/2HCO₃ co-transporter at the apical membrane of frog and bovine RPE (Hughes et al., 1989; Kenyon et al., 1997). In addition, this Na/2HCO₃ co-transporter (NBC1) has been immunolocalized to the apical membrane of rat RPE (Bok et al., 2001). Since CO₂ is an acid, and HCO₃⁻ is a base, it is possible that CO₂-entry into the cell is balanced by concomitant HCO₃⁻-entry at the apical membrane, thus maintaining pHᵢ homeostasis of the RPE. This notion is further discussed in the next section.
Section 3.1 conclusion: RPE apical membrane is more CO₂-permeable than its basolateral membrane

<table>
<thead>
<tr>
<th>Supporting experimental observations</th>
<th>Interpretation</th>
<th>Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>13% apical CO₂ causes 10-fold larger acidification than 13% basal CO₂</td>
<td>Apical membrane is more CO₂-permeable than basolateral membrane</td>
<td>3-1</td>
</tr>
<tr>
<td>1% apical CO₂ causes 10-fold larger acidification than 1% basal CO₂</td>
<td>Apical membrane is more CO₂-permeable than basolateral membrane</td>
<td>3-2</td>
</tr>
<tr>
<td>Damaging RPE monolayer decreases 13% apical CO₂-induced acidification</td>
<td>The polycarbonate porous membrane does not limit CO₂-diffusion</td>
<td>3-4</td>
</tr>
<tr>
<td>Damaging RPE monolayer increases 13% basal CO₂-induced acidification</td>
<td>Apical membrane is more CO₂-permeable than basolateral membrane</td>
<td>3-5</td>
</tr>
<tr>
<td>Stopping apical flow increases 13% basal CO₂-induced acidification</td>
<td>Differences apical/basal bath perfusion rate did not affect CO₂-permeability</td>
<td>N/A</td>
</tr>
<tr>
<td>Mounting RPE monolayer upside-down did not affect 13% CO₂-induced responses</td>
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<td></td>
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</tbody>
</table>
Section 3.3 – HCO₃ transport at the apical membrane

To test the activity of apical NBC1 in cultured hRPE, we added DIDS (NBC1 inhibitor) to the apical bath and compared the resultant pHᵢ and TEP responses in control Ringer (26.2 mM HCO₃) vs. low HCO₃ Ringer (2.62 mM HCO₃) in the apical bath (Fig. 3-7).

Fig. 3-7: Apical DIDS induced pHᵢ, TEP, and RT responses in the presence of low [HCO₃] Ringer (2.62 mM) in the apical bath.

NBC1, like any transporter, is driven by the concentration gradient of its substrate (i.e., Na and HCO₃). Normally, the large inward Na-gradient drives Na/2HCO₃ transport into the cell. Data from six experiments showed that in control Ringer, apical DIDS acidified the cell by 0.05 ± 0.02 and decreased TEP by 1.59 ± 0.63 mV. When [HCO₃] in the apical bath was reduced 10-fold (from 26.2 mM to 2.62 mM), the resultant HCO₃-
gradient reverses NBC1 to transport NaHCO\textsubscript{3} out of the cell. In this condition, we found that adding DIDS to the apical bath alkalinized the cell by 0.04 ± 0.01 and transiently increased TEP by 0.30 ± 0.15 mV. These apical DIDS-induced pHi and TEP responses are consistent with the inhibition of an electrogenic HCO\textsubscript{3}-dependent mechanism in the forward or reverse direction.

Reducing apical bath [HCO\textsubscript{3}] decreased pH\textsubscript{i} and TEP – these responses reflect a change in Na/2HCO\textsubscript{3}-transport activity and we can use this maneuver to further study NBC1 mediated HCO\textsubscript{3}-transport. To test this possibility, we decreased apical bath [HCO\textsubscript{3}] 10-fold and compared the resultant pH\textsubscript{i} and TEP responses in the presence or absence of apical DIDS (Fig. 3-8).

![Fig. 3-8: Apical bath Δ[HCO₃⁻]-induced pHi, TEP, and Rₜ responses in the presence of apical DIDS.](image)
In three experiments, DIDS reduced the apical bath $\Delta[HCO_3^-]$-induced TEP response 7-fold (from $\Delta$TEP = 2.1 ± 0.2 mV to 0.3 ± 0.2 mV; p < 0.01). The effect of DIDS on the TEP response was partially reversible following a five-minute washout ($\Delta$TEP = 1.28 ± 0.22 mV). This result indicates that apical DIDS almost completely blocked the apical membrane Na/2HCO$_3$ co-transporter activity. Surprisingly, the apical bath $\Delta[HCO_3^-]$-induced acidification ($\Delta$pHi = 0.10 ± 0.02) was not significantly affected by DIDS ($\Delta$pHi = 0.09 ± 0.01; n = 3; p > 0.05), suggesting the presence of a DIDS-insensitive HCO$_3$-transporter at the apical membrane.

Besides pNBC1 (SLC4A4; GeneID: 8671), a DIDS-insensitive and electroneutral Na/HCO$_3$ co-transporter, NBC3/NBCn1 (SLC4A7; GeneID: 9497), is also highly expressed at the apical membrane of human RPE (Wang and Miller, ARVO 2007, #6034; Zhi et al., ARVO 2007, #2532). Our observation that apical DIDS had little effect on the apical bath $\Delta[HCO_3^-]$-induced acidification suggests that NBC3 is highly active in the RPE. However, this does not indicate that NBC1 has a lower activity than NBC3 because NBC1 is electrogenic, and is therefore limited by both the membrane voltage and HCO$_3$-gradient. In contrast, NBC3 is limited only by the HCO$_3$-gradient. Thus the relative activities of NBC1 and NBC3 cannot be accurately evaluated by comparing the apical bath $\Delta[HCO_3^-]$-induced pH$_i$ responses in the presence vs. absence of apical DIDS.

The presence of carbonic anhydrase II (CA II) and several apical membrane-bound carbonic anhydrases (e.g., CAs IV, IX, XII, and XIV) support the notion of HCO$_3$-mediated CO$_2$ transport from the SRS into the RPE via the electrogenic Na/nHCO$_3$ co-transporter.
transporter as illustrated in Fig 2-4. According to the bicarbonate transport metabolon theory (Sterling et al., 2001; Alvarez et al., 2003), a membrane bound CA physically binds and interacts with HCO3-transporters at the apical membrane (e.g., NBC1 and NBC3). If this synergistic form of HCO3-transport exists in RPE, increasing CO2 level at the apical surface and in the vicinity of the active site of transmembrane CAs should stimulate the conversion of CO2 and H2O into H+ and HCO3. The resultant increase in local [HCO3] near the ion-pore of the HCO3-transporter enhances HCO3-entry into the cell. In the remainder of this section, we present experiments to evaluate this possibility.

Our first step is to determine if increasing apical bath CO2 stimulates HCO3-entry into the cell via NBC1. To test this hypothesis, we compared apical DIDS (0.5 mM) induced pHi and TEP responses in control (5% CO2) Ringer to that in 1% or 13% CO2 equilibrated
Ringer (Fig. 3-9). If NBC1 activity is enhanced by an increased CO₂ level in the apical bath, then DIDS-induced inhibition of NBC1 should cause larger pHᵢ and TEP responses in 13% apical CO₂ than in control (5% CO₂). Alternatively, if decreasing apical bath CO₂ from 5% to 1% decreased NBC1 activity, the DIDS-induced pHᵢ and TEP responses should be smaller in 1% apical CO₂ than in control. In four experiments, apical DIDS induced pHᵢ and TEP responses in control (5% CO₂) Ringer (ΔpHᵢ = 0.05 ± 0.02; ΔTEP = 1.52 ± 0.33 mV) were the same as that in 13% CO₂ equilibrated Ringer (ΔpHᵢ = 0.05 ± 0.02; ΔTEP = 1.57 ± 0.67 mV; p > 0.05). Similarly, the apical DIDS induced pHᵢ and TEP responses in control Ringer (ΔpHᵢ = 0.05 ± 0.02; ΔTEP = 1.66 ± 0.59 mV) were the same as that in 1% CO₂ equilibrated Ringer (ΔpHᵢ = 0.06 ± 0.02; ΔTEP = 1.31 ± 0.78 mV; n = 5; p > 0.05; Fig. 3-10).

Fig. 3-10: DIDS induced pHᵢ, TEP, and Rᵣ responses in the presence of 1% apical CO₂.
To further test the pH$_i$-sensitivity of the apical membrane Na/2HCO$_3$ co-transporter, we perfused 13% CO$_2$ equilibrated Ringer into the apical bath in the presence or absence of 0.5 mM apical DIDS (Fig. 3-11). If increasing apical bath CO$_2$ stimulates NBC1, 13% apical CO$_2$ should cause a larger acidification in the absence of NBC1 activity.

However, in the presence of apical DIDS, the 13% CO$_2$-induced acidification ($\Delta$pH$_i = 0.22 \pm 0.03$) was the same as control ($\Delta$pH$_i = 0.22 \pm 0.02$; n = 4; p > 0.05). Taken together, these observations (Fig. 3-9, -10, -11) lead to the conclusion that Na/2HCO$_3$ co-transport activity is unaffected by apical bath CO$_2$. This is probably due to the high CO$_2$-permeability of the apical membrane; CO$_2$/HCO$_3$ equilibration across the apical
membrane is achieved very quickly, which prevented the formation of a HCO3-gradient
needed to increase the rate of NaHCO3 transport via NBC1.

According to Fig. 1-4, HCO3 that enters the RPE via NBC1 can be converted by CA II
into CO2 and H2O in the cytosol. By effectively removing HCO3 (by converting it to
CO2) near NBC1’s ion-pore in the cytosol, CA II facilitates apical Na/2HCO3 co-
transport activity by maintaining a high local [HCO3] gradient across the transporter. We
test this notion by decreasing apical bath [HCO3] (10-fold) and comparing the resultant
pHi and TEP responses in the presence of 250 µM apical dorzolamide (DZA; CA II
inhibitor) to that in control (Fig. 3-12).

Fig. 3-12: Apical bath △[HCO3]-induced pHi, TEP, and R΄ responses in the presence of apical
dorzolamide.
In five experiments, DZA decreased apical bath $\Delta[HCO_3^-]$-induced TEP response by 60% (from $2.25 \pm 0.81$ to $0.89 \pm 0.29$ mV; $p < 0.01$) and increased the pH$_i$ response from $0.11 \pm 0.01$ to $0.19 \pm 0.01$ ($p < 0.01$) – the effect of DZA on these responses were partially reversible following washout ($\Delta$TEP = $1.27 \pm 0.46$ mV; $\Delta$pH$_i$ = $0.17 \pm 0.02$). The reduced apical bath $\Delta[HCO_3^-]$-induced TEP response in the presence of DZA indicates inhibition of apical Na/2HCO$_3$ co-transport activity. On the other hand, the apical bath $\Delta[HCO_3^-]$-induced acidification was larger in the presence of DZA because CA II-inhibition reduces intracellular CO$_2$/HCO$_3$ buffering capacity, which compromises the ability of the RPE to buffer the acidification caused by HCO$_3$-efflux from the apical membrane. This experiment indicates that NBC1 activity is dependent on CA II activity.

In summary, increasing apical bath CO$_2$ did not stimulate NaHCO$_3$ transport via NBC1. This result may seem counter intuitive in the context of the HCO$_3$-transport metabolon (see Fig. 1-9), but it can be understood by taking into account the higher apical membrane CO$_2$ permeability than the basolateral membrane; 13% CO$_2$ in the apical bath equilibrates very quickly in the cytosol. The lower CO$_2$ permeability of the basolateral membrane causes CO$_2$ to accumulate in the cytosol, thus allowing CO$_2$ level in the cytosol to approximately equal that in the apical bath. Without a large CO$_2$ gradient across the apical membrane, little or no CO$_2$ is converted into HCO$_3$ for NBC1-mediated HCO$_3$-transport. In contrast, 13% apical CO$_2$ generates a large CO$_2$ gradient across the basolateral membrane, which should drive carbonic anhydrase-mediated conversion of CO$_2$ to HCO$_3$ for HCO$_3$-transport across the basolateral membrane (see next section).
Section 3.2 Conclusions: HCO₃⁻ is transported across the apical membrane via the electrogenic and DIDS-sensitive Na⁺/2HCO₃⁻ co-transporter (NBC1).

NBC1 activity is unaffected by CO₂-gradient (13% apical CO₂)

NBC1 activity is dependent on carbonic anhydrase II activity

Supporting experimental observations
- Apical DIDS increased pH, in control (26 mM HCO₃⁻), but decreased pH in low HCO₃⁻ Ringer (2.6 mM)
- Apical DIDS decreased TEP in control (26 mM HCO₃⁻), but increased TEP in low HCO₃⁻ Ringer (2.6 mM)
- Decreasing apical bath [HCO₃⁻] decreased pH
- Apical bath Δ[HCO₃⁻]-induced acidification is unaffected by apical DIDS
- Decreasing apical bath [HCO₃⁻] decreased TEP
- Apical bath Δ[HCO₃⁻]-induced TEP response is blocked by apical DIDS
- Apical DIDS-induced pH, and TEP responses were the same in 5%, 1% or 13% apical CO₂
- 13% apical CO₂-induced pH response were unaffected by apical DIDS
- Apical bath Δ[HCO₃⁻]-induced TEP response was reduced by apical dorzolamide

<table>
<thead>
<tr>
<th>Interpretation</th>
<th>Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCO₃⁻ is transported across the apical membrane via HCO₃⁻-transporter</td>
<td>3-7</td>
</tr>
<tr>
<td>HCO₃⁻ is transported across the apical membrane via an electrogenic HCO₃⁻-transporter</td>
<td>3-7</td>
</tr>
<tr>
<td>HCO₃⁻ is transported across the apical membrane via NBC1 and/or NBC3</td>
<td>3-8</td>
</tr>
<tr>
<td>HCO₃⁻ is transported across the apical membrane via NBC3</td>
<td>3-8</td>
</tr>
<tr>
<td>HCO₃⁻ is transported across the apical membrane via an electrogenic HCO₃⁻-transporter</td>
<td>3-8</td>
</tr>
<tr>
<td>HCO₃⁻ is transported across the apical membrane via NBC1</td>
<td>3-8</td>
</tr>
<tr>
<td>CO₂-gradient or cell acidification did not affect NBC1 activity</td>
<td>3-9 &amp; 3-10</td>
</tr>
<tr>
<td>CO₂-gradient or cell acidification did not affect NBC1 activity</td>
<td>3-11</td>
</tr>
<tr>
<td>NBC1 activity is dependent on cytosolic carbonic anhydrase II</td>
<td>3-12</td>
</tr>
</tbody>
</table>
Section 3.4 – HCO3-transport at RPE basolateral membrane

In frog RPE, a DIDS-sensitive Cl/HCO3 exchanger was found at the basolateral membrane (Lin & Miller, 1994). The activity of this Cl/HCO3 exchanger is pH dependent; it was inhibited by acid and activated by base. Normally, the Cl/HCO3 exchanger uses the Cl-gradient (inward) to drive HCO3 out of the cell. We can evaluate basolateral membrane Cl/HCO3 exchanger activity in hfRPE by reducing basal bath [Cl] (from 126 mM to 1 mM). This creates a large outward Cl gradient across the basolateral membrane that drives HCO3 into the cell to cause an alkalinization ($\Delta pHi = 0.22$; Fig. 3-13). In three experiments this alkalinization ($\Delta pHi = 0.18 \pm 0.05$) was abolished by 0.5 mM basal DIDS ($\Delta pHi = 0.02 \pm 0.01$; n = 3; $p < 0.05$).

Fig. 3-13: Basal bath $\Delta$[Cl]-induced $pHi$, TEP, and $R_T$ responses in the presence of basal DIDS.
Next, we tested the pH$_i$-dependence of the Cl/HCO$_3^-$ exchanger by comparing the basal bath Δ[Cl]-induced pH$_i$ response in 5% vs. 13% apical bath CO$_2$ (Fig. 3-14). The steady-state pH$_i$ in 5% and 13% apical bath CO$_2$ differed significantly, which required us to use the total buffering capacity of the hfRPE to calculate equivalent H$^+$-fluxes. In the presence of 13% CO$_2$ equilibrated Ringer in the apical bath, the basal bath Δ[Cl]-induced change in H$^+$-flux was 2.3 ± 1.0 mM·min$^{-1}$, ≈ 4-fold smaller than the H$^+$-flux in 5% CO$_2$ (9.0 ± 4.5 mM·min$^{-1}$; n = 7; p < 0.01); this effect was fully reversible. Fig. 3-15 shows a parallel experiment in which basal bath [Cl] was reduced in the presence of 1% CO$_2$ equilibrated Ringer in the apical bath.
In 1% apical bath CO₂, the basal bath Δ[Cl]-induced proton flux was 27.4 ± 10.8 mM·min⁻¹, or ≈ 5-fold larger than the flux in 5% CO₂ (5.9 ± 6.5 mM·min⁻¹; n = 5; p = 0.01). These experiments indicate that the DIDS-sensitive basolateral membrane Cl/HCO₃ exchanger in hfRPE is pH-independent. From our laboratory’s Affymetrix data, AE2 (SLC4A2; GeneID: 6522) is the only AE isoform detected in cultured fetal human RPE and in native adult and fetal human RPE. Since AE2 is pH-sensitive (Kurschat et al., 2006; Stewart et al., 2007), it is possibly the isoform located at the basolateral membrane of hfRPE.

In the retina, the transition from light to dark is followed by increased metabolism and CO₂ and water release into the subretinal space. In section 2 of this chapter, we
hypothesized that the relatively higher apical vs. basolateral membrane CO₂-permeability could produce a large CO₂-gradient across the basolateral membrane *in vivo*. With the help of carbonic anhydrase activity, this CO₂-gradient can drive HCO₃⁻ out of the basolateral membrane via HCO₃⁻-transporters. Although the Cl/HCO₃ exchanger (AE2) is a HCO₃⁻-transporter, our experiments (Fig. 3-13) show that AE2 is inhibited by 13% apical CO₂. To facilitate an increased HCO₃⁻-efflux from the basolateral membrane, the RPE requires an alternate HCO₃⁻-efflux pathway. In this regard, evidence of an electrogentic Na/nHCO₃ co-transporter at the basolateral membrane has been reported in bovine RPE (Kenyon *et al.*, 1997). This transporter may work in parallel with AE2 to mediate HCO₃⁻-efflux at the basolateral membrane.

To test the basolateral membrane Na/nHCO₃ co-transporter activity in hfRPE, we reduced basal bath [HCO₃⁻] by 10-fold to induce a large [HCO₃⁻]-gradient across the basolateral membrane. This maneuver causes HCO₃⁻ to leave the cell via both AE2 and the Na/nHCO₃ co-transporter, resulting in intracellular acidification. Since this Na/nHCO₃ co-transporter is an electrogentic mechanism, HCO₃⁻-efflux via this transporter will depolarize the basolateral membrane, which increases TEP. In contrast, AE2 is electroneutral (no transfer of net ionic charges across the membrane) and therefore could not have contributed to the TEP response; the basal bath Δ[HCO₃⁻]₀ induced TEP response originates solely from Na/nHCO₃ co-transport activity. In confluent monolayers of hfRPE, reducing basal bath [HCO₃⁻] 10-fold (5% CO₂) acidified the cells by 0.20 ± 0.05 with an equivalent H⁺-flux of 6.2 ± 1.5 mM·min⁻¹ (n = 45), and increased TEP by 1.18 ± 0.60 mV (n = 53).
Table 3-1. Summary of basal bath $\Delta[HCO_3^-]$-induced $\Delta pHi$ responses.

<table>
<thead>
<tr>
<th>inhibitor/condition$^a$</th>
<th>Apical Bath</th>
<th>Basal Bath</th>
<th>$\Delta pHi$</th>
<th>$H^+$-flux</th>
<th>$\Delta pHi$</th>
<th>$H^+$-flux</th>
<th>$p^c$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIDS</td>
<td>Na-free</td>
<td>$\Delta pHi$</td>
<td>-0.20 ± 0.04</td>
<td>-6.5 ± 1.2</td>
<td>-0.09 ± 0.05</td>
<td>-3.6 ± 2.7</td>
<td>-0.12 ± 0.03</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$H^+$-flux</td>
<td>-7.2 ± 1.7</td>
<td></td>
<td>-2.8 ± 0.7</td>
<td></td>
<td>-4.3 ± 1.3</td>
<td>S</td>
</tr>
<tr>
<td>Na-free</td>
<td>DIDS</td>
<td>$\Delta pHi$</td>
<td>-0.21 ± 0.04</td>
<td>-7.2 ± 1.7</td>
<td>-0.08 ± 0.01</td>
<td>-2.8 ± 0.7</td>
<td>-0.14 ± 0.02</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$H^+$-flux</td>
<td>-7.1 ± 1.3</td>
<td></td>
<td>-8.3 ± 1.6</td>
<td></td>
<td>-5.7 ± 1.4</td>
<td>S</td>
</tr>
<tr>
<td>amiloride</td>
<td>Na-free</td>
<td>$\Delta pHi$</td>
<td>-0.20 ± 0.03</td>
<td>-7.1 ± 1.3</td>
<td>-0.21 ± 0.03</td>
<td>-8.3 ± 1.6</td>
<td>-0.22 ± 0.03</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$H^+$-flux</td>
<td>-6.9 ± 1.8</td>
<td></td>
<td>-6.8 ± 0.9</td>
<td></td>
<td>-7.6 ± 1.6</td>
<td>NS</td>
</tr>
<tr>
<td>bumetanide</td>
<td>DIDS</td>
<td>$\Delta pHi$</td>
<td>-0.18 ± 0.02</td>
<td>-5.1 ± 2.1</td>
<td>-0.20 ± 0.02</td>
<td>-5.4 ± 3.2</td>
<td>-0.23 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$H^+$-flux</td>
<td>-5.0 ± 1.1</td>
<td></td>
<td>-6.0 ± 1.1</td>
<td></td>
<td>-5.7 ± 0.9</td>
<td>S</td>
</tr>
<tr>
<td>ouabain</td>
<td>13% CO₂</td>
<td>$\Delta pHi$</td>
<td>-0.19 ± 0.03</td>
<td>-5.2 ± 0.3</td>
<td>-0.18 ± 0.03</td>
<td>-5.1 ± 0.7</td>
<td>-0.21 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$H^+$-flux</td>
<td>-6.0 ± 1.3</td>
<td></td>
<td>-6.1 ± 2.3</td>
<td></td>
<td>-5.6 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>DIDS</td>
<td>1% CO₂</td>
<td>$\Delta pHi$</td>
<td>-0.24 ± 0.08</td>
<td>-7.2 ± 1.8</td>
<td>-0.20 ± 0.06</td>
<td>-10.7 ± 1.9</td>
<td>-0.20 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$H^+$-flux</td>
<td>-7.0 ± 0.08</td>
<td></td>
<td>-10.7 ± 1.9</td>
<td></td>
<td>-6.0 ± 1.3</td>
<td>S</td>
</tr>
</tbody>
</table>

a. Blank cells indicate that control Ringer was perfused into the corresponding bath.
b. $H^+$-flux has units of mM·min$^{-1}$ and all values are reported as mean ± SD.
c. Student’s t-test for statistical significance between basal bath $\Delta[HCO_3^-]$-induced $\Delta pHi$ response in control vs. in the presence of inhibitor/condition. “S” indicates statistical significance (p < 0.05), “NS” indicates statistical insignificance (p > 0.05).
Table 3-2. Summary of basal bath Δ[HCO_3^-]-induced TEP responses.

<table>
<thead>
<tr>
<th>inhibitor/condition</th>
<th>2.62 mM basal bath [HCO_3^-]-induced TEP response (mV)^b</th>
<th>p^c</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical</td>
<td>Basal Control w/ inhibitor Recovery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIDS</td>
<td>1.41 ± 0.69 0.42 ± 0.29 0.56 ± 0.25 S 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na-free</td>
<td>1.01 ± 0.21 0.04 ± 0.07 0.79 ± 0.38 S 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>amiloride</td>
<td>1.28 ± 0.58 1.24 ± 0.50 1.23 ± 0.40 NS 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bumetanide</td>
<td>0.89 ± 0.28 0.80 ± 0.18 0.97 ± 0.29 NS 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ouabain</td>
<td>1.26 ± 0.59 1.17 ± 0.49 1.19 ± 0.51 NS 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIDS</td>
<td>0.86 ± 0.16 0.49 ± 0.07 0.63 ± 0.15 S 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DZA</td>
<td>1.44 ± 0.80 0.98 ± 0.50 1.06 ± 0.55 S 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13% CO_2</td>
<td>1.11 ± 0.67 1.35 ± 0.78 1.07 ± 0.67 S 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% CO_2</td>
<td>1.26 ± 0.74 0.37 ± 0.32 0.99 ± 0.59 S 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Blank cells indicate that control Ringer was perfused into the corresponding bath.
b. All ΔTEP values are reported as mean ± SD.
c. Student’s t-test for statistical significance between basal bath Δ[HCO_3^-]-induced TEP response in control vs. in the presence of inhibitor/condition. “S” indicates statistical significance (p < 0.05), “NS” indicates statistical insignificance (p > 0.05).
Electrogenic members of the NBC family of Na/HCO₃ transporters (e.g., NBC1 and NBC4) are DIDS sensitive. To ensure that the basal bath Δ[HCO₃] -induced pHᵢ and TEP responses are mediated by a Na/nHCO₃ co-transporter, we compared these pHᵢ and TEP responses in the presence vs. absence of 0.5 mM DIDS in the basal bath (Fig. 3-16).

In five experiments, basal DIDS reduced the basal bath Δ[HCO₃] -induced acidification by ≈ 50% (Table 3-1), and reduced the TEP response by ≈ 70% (Table 3-2). The inhibitory effect of DIDS on the pHᵢ and TEP responses was irreversible (5 min washout). The significant inhibition of the basal bath Δ[HCO₃] -induced acidification by basal DIDS indicates that HCO₃-transporters at the basolateral membrane are DIDS-
sensitive. Since TEP respond only to electrogenic processes, the significant inhibition of the TEP response by basal DIDS indicates a reduction in Na/nHCO₃ co-transport activity.

Since the Na/nHCO₃ co-transporter is a Na-dependent mechanism, the removal of Na from both apical and basal baths should eliminate its activity. Therefore, we reduced basal bath [HCO₃⁻] and measure the resultant pHᵢ and TEP responses in the presence or absence of Na (Fig. 3-17).

Fig. 3-17: Basal bath Δ[HCO₃⁻]-induced pHᵢ, TEP, and Rₜ responses in the absence of Na⁺.

When Na is removed from both apical and basal baths, the cell acidified by ~ 0.3 pH-units. This observation is consistent with HCO₃⁻-efflux from both the apical and basolateral membranes via Na-linked HCO₃ transporters. In three experiments, Na-removal reduced the basal bath Δ[HCO₃⁻]-induced acidification by more than 2-fold
compared to control (Table 3-1). In addition, the basal bath $\Delta$[HCO$_3$]-induced TEP response was essentially abolished in the absence of Na, and this effect was reversible (Table 3-2). These experiments indicate that although reducing basal bath [HCO$_3$] causes HCO$_3$-efflux via both AE2 and Na/nHCO$_3$ co-transporter, the TEP response corresponds specifically to Na/nHCO$_3$ co-transporter activity due to its electrogenicity and Na-dependence. This allows one to distinguish the activity of the Na/nHCO$_3$ co-transporter from that of AE2.

Affymetrix data on human RPE (native adult and fetal RPE, and cultured fetal RPE) (Wang and Miller, ARVO 2007, #6034) show high mRNA expression levels for NBC1 (SLC4A4; GeneID: 8671) and NBC4/NBCe2 (SLC4A5; GeneID: 57835), both of which are candidates for the identity of the basolateral membrane Na/nHCO$_3$ co-transporter in human RPE. Although this co-transporter’s identity is unknown, both NBC1 and NBC4 have been shown to transport Na:HCO$_3$ with a stoichiometry of 1:2 (Hughes et al., 1989; Gross et al., 2001; Virkki et al., 2002), suggesting inward Na/HCO$_3$ co-transport from the basolateral membrane. However, NBC4 transports Na:HCO$_3$ with a 1:3 stoichiometry at the apical membrane of the choroid plexus epithelium (Millar & Brown, 2008). Since both the RPE and the choroid plexus epithelium derives from the neural ectoderm and share many similarities in HCO$_3$-transport mechanisms (Brown et al., 2004; Praetorius, 2007), it is possible that the RPE expresses NBC4 at the basolateral membrane and transports Na/nHCO$_3$ with a 1:3 Na:HCO$_3$ stoichiometry. In addition, our calculation of the reversal potential of the Na/nHCO$_3$ co-transporter indicate that a 1:3 stoichiometry is
required for Na/HCO₃ transport out of the cell; this calculation is based on our estimation of resting [Na]ᵢ and pHᵢ in control Ringer with the following equation:

\[
E_{\text{NBC}} = \frac{2.3RT}{F(n-1)} \log \left( \frac{[\text{Na}^+]_{\text{in}}}{[\text{Na}^+]_{\text{out}}} \left( \frac{[\text{HCO}_3^-]_{\text{in}}}{[\text{HCO}_3^-]_{\text{out}}} \right)^n \right),
\]

where \([\text{Na}^+]_{\text{in}} = 15.7 \text{ mM}, [\text{Na}^+]_{\text{out}} = 143.7 \text{ mM}, [\text{HCO}_3^-]_{\text{in}} = 27.9 \text{ mM}, [\text{HCO}_3^-]_{\text{out}} = 26.2 \text{ mM}. \ n \ is \ the \ stoichiometry \ of \ the \ Na/nHCO₃ \ co-transporter. \ For \ a \ Na:HCO₃ \ transport \ stoichiometry \ of \ 1:2 \ and \ 1:3, \ we \ calculated \ the \ reversal \ potential \ of \ the \ Na/nHCO₃ \ co-transporter \ NBC (E_{\text{NBC}}) \ to \ be \ -55.7 \ and \ -27 \ mV \ respectively. \ To \ transport \ Na/nHCO₃ \ out \ of \ the \ cell \ against \ the \ strong \ inward \ Na-gradient \ in \ control \ conditions, \ E_{\text{NBC}} \ must \ be \ more \ depolarized \ than \ V_B (49.8 ± 3.7 \text{ mV}; Maminishkis et al, 2006) \ and \ this \ condition \ is \ achieved \ for \ a \ Na:HCO₃ \ transport \ stoichiometry \ of \ 1:3. \ The \ isoform \ of \ this \ basolateral \ membrane \ NBC \ has \ not \ been \ determined.

When apical bath CO₂ is increased, CO₂ diffuses into the cell and accumulates within the cytosol, which shifts the CO₂/HCO₃ equilibrium towards the formation of HCO₃⁻ and H⁺ (catalyzed by carbonic anhydrase II). In this section, we showed that 13% apical CO₂ acidified the cell and inhibited the Cl/HCO₃ exchanger (AE2) at the basolateral membrane, indicating that AE2 cannot serve as the main HCO₃⁻-efflux pathway when apical bath CO₂ increases. However, we also confirmed the presence and activity of an alternate HCO₃⁻-efflux pathway at the basolateral membrane – Na/nHCO₃ co-transporter (NBC). In the next section, we investigate if increasing apical bath CO₂ can stimulate this Na/nHCO₃ co-transporter.
Section 3.5 – Basolateral membrane NBC: dependence on CO₂ and HCO₃

Carbonic anhydrase II (CA II) catalyzes the conversion of CO₂ into HCO₃, which is subsequently transported out of the basolateral membrane via the Na/nHCO₃ co-transporter (NBC). Since optimal HCO₃-transport requires a steady supply of HCO₃, inhibition of CA II by dorzolamide (DZA) should reduce Na/nHCO₃ co-transport across the basolateral membrane. This notion was tested by reducing basal bath [HCO₃] 10-fold in the presence of DZA (250 µM) in the basal bath (Fig. 3-18).

In a total of nine experiments, DZA reduced the basal bath Δ[HCO₃]-induced TEP response by ≈ 30% (Table 3-2) suggesting that CA II-inhibition reduces basolateral...
membrane NBC activity. In contrast, DZA did not affect the basal bath $\Delta$[HCO$_3$]-induced acidification (Table 3-1). This lack of effect probably occurred because the DZA-induced reduction in basolateral membrane HCO$_3$ efflux is counteracted by a concomitant reduction in intracellular CO$_2$/HCO$_3$ buffering capacity.

A 13% CO$_2$-load applied to the apical membrane should increase basolateral membrane NBC activity by shifting intracellular CO$_2$/HCO$_3$ equilibrium towards the formation of HCO$_3$ (facilitated by CA II activity). To test this hypothesis, we made a 10-fold reduction in basal bath [HCO$_3$] (from 26.2 mM to 2.62 mM) and compared the resultant pH$_i$ and TEP responses in 5% vs. 13% apical bath CO$_2$ (Fig. 3-19).

Fig. 3-19: Basal bath $\Delta$[HCO$_3$]-induced pH$_i$, TEP, and $R_T$ responses with 13% apical CO$_2$. 
With 13% CO₂ in the apical bath, decreasing basal bath [HCO₃⁻] induced a TEP response that was ≈ 20% larger than control (Table 3-2). However, there was no change in basal bath Δ[HCO₃⁻]-induced pHᵢ response (ΔpHᵢ and H⁺-flux) in the presence of 5% or 13% apical bath CO₂ (Table 3-1). Presumably, 13% apical CO₂ did not significantly alter H⁺-flux caused by basal bath Δ[HCO₃⁻] because the CO₂-induced increase in HCO₃-efflux via the Na/nHCO₃ co-transporter was offset by concomitant inhibition of the pHᵢ-sensitive Cl/HCO₃ exchanger, thus producing no observable change in net H⁺-flux. In similar experiments, we reduced basal bath [HCO₃⁻] in 1% apical bath CO₂ (Fig. 3-20).

Fig. 3-20: Basal bath Δ[HCO₃⁻]-induced pHᵢ, TEP, and Rₜ responses with 1% apical CO₂.

This maneuver should reduce free-HCO₃ in the cell and subsequently decrease basolateral membrane NBC activity. In 1% apical CO₂, the basal bath Δ[HCO₃⁻]-induced...
TEP response was more than 5-fold smaller than in 5% apical CO₂ (Table 3-2). This result indicates that intracellular CO₂ is a significant source of HCO₃-supply for the basolateral membrane NBC. 1% apical CO₂ increased the basal bath $\Delta$[HCO₃]-induced equivalent H⁺-flux from 7.2 ± 1.8 to 10.7 ± 1.9 mM·min⁻¹ ($n = 5$; $p = 0.04$). The H⁺-flux in the presence of 1% apical bath CO₂ was larger probably because the resultant alkalization activated the Cl/HCO₃ exchanger more than the reduction in Na/nHCO₃ co-transport activity.

By showing that 13% and 1% apical CO₂ increased and decreased the basal bath $\Delta$[HCO₃]-induced TEP response respectively, we confirmed our hypothesis that an increase in apical bath CO₂ stimulates HCO₃ efflux from the basolateral membrane via the basolateral membrane NBC. As shown in Fig. 3-18, this process is facilitated by cytosolic CA II activity. Since 13% apical CO₂ stimulates NaHCO₃ efflux from the basolateral membrane, we should observe a decrease in intracellular [Na]. In addition, since Na is a substrate of the basolateral membrane NBC, we expect its activity to depend on one or more Na-transport mechanism as its main source of Na. These mechanisms are explored in the next few sections.
Sections 3.3 & 3.4 conclusions: Basolateral membrane HCO₃⁻-transport is mediated by a Cl⁻/HCO₃⁻ exchanger (AE2) and an electroneutral Na⁺/HCO₃⁻ co-transporter (NBC).

HCO₃⁻-transport via basolateral membrane NBC is dependent on carbonic anhydrase II activity.

Increasing apical bath CO₂ stimulates HCO₃⁻-transport via basolateral membrane NBC.

Supporting experimental observations:
- Decreasing basal bath [Cl⁻] increased pH, and this alkalinization is blocked by basal DIDS.
- Basal bath Δ[Cl⁻] induced alkalinization is inhibited and activated in 13% and 1% apical CO₂, respectively.
- Decreasing basal bath [HCO₃⁻] decreased pH, and this acidification is reduced by basal DIDS.
- Decreasing basal bath [HCO₃⁻] increased TEP, and this response is blocked by basal DIDS.
- Increasing basal bath [HCO₃⁻] increased TEP, and this acidification is reduced in the absence of Na⁺.
- Basal bath Δ[HCO₃⁻] induced TEP response is abolished by Na⁺-removal.

Interpretation:
- HCO₃⁻ is transported across the basolateral membrane via AE2: Fig. 3-13
- AE2-mediated HCO₃⁻-transport is pH-dependent: Fig. 3-14 & 3-15
- HCO₃⁻ is transported at the basolateral membrane via both AE2 and NBC: Fig. 3-16
- Electroneutral HCO₃⁻-transport at the basolateral membrane is DIDS-sensitive: Fig. 3-17
- HCO₃⁻ is transported at the basolateral membrane via both AE2 and NBC: Fig. 3-17
- Electroneutral HCO₃⁻-transport at the basolateral membrane is Na⁺-dependent: Fig. 3-18
- HCO₃⁻-transport via basolateral membrane NBC is dependent on CA II activity: Fig. 3-19
- Increasing apical bath CO₂ stimulates basolateral membrane NBC: Fig. 3-20
- Decreasing apical bath CO₂ stimulates basolateral membrane NBC: Fig. 3-20
Section 3.6 – CO₂ and Na-transport

We showed that 13% apical CO₂ increased the basal bath Δ[HCO₃⁻]-induced TEP response (Fig. 3-19), suggesting that 13% apical CO₂ activates the basolateral membrane Na/nHCO₃ co-transporter, which should decrease [Na]ᵢ. To test this notion, we observed the change in intracellular [Na] (with Na-sensitive dye) when apical bath CO₂ was increased from 5% to 13% (Fig. 3-21). Surprisingly, 13% apical CO₂ increased [Na]ᵢ from 15.7 ± 3.3 to 24.0 ± 5.3 mM (n = 6; p < 0.05).

![Fig. 3-21: 13% apical or basal CO₂ induced [Na]ᵢ responses.](image)

This suggests that one or more Na-entry pathways at the apical membrane are affected by 13% apical CO₂ to produce the observed increase in [Na]ᵢ. Early studies showed that the Na/H exchanger can be activated by intracellular acidification (Aronson et al., 1982; Dunham et al., 2004). Therefore it is possible that 13% apical CO₂ induced acidification stimulated NHE activity, which drives Na into the cell – this may explain the 13% apical CO₂ induced increase in [Na]ᵢ. To test whether 13% apical CO₂-induced acidification activated NHE, we compared the effect of 1 mM amiloride (NHE inhibitor) on the
steady-state pH, of the RPE in control Ringer (5% CO₂) to that in 13% CO₂ equilibrated Ringer (Fig. 3-22). If 13% apical CO₂ stimulated NHE activity, its inhibition by amiloride should cause a large acidification. However in three experiments, adding 1 mM amiloride into the apical bath did not cause any change in steady-state pH, in either 5% or 13% apical bath CO₂.

As an additional test, we compared the magnitude of the 13% apical CO₂-induced acidification in the presence or absence of 1 mM amiloride in the apical bath (Fig. 3-23). If NHE activity (H⁺-efflux) helps buffer the 13% CO₂ induced acidification, this acidification should be significantly larger in the presence of apical amiloride.

Fig. 3-22: Apical amiloride-induced pH, TEP, and Rₜ responses with 13% apical CO₂.
Fig. 3-23: 13% apical CO$_2$-induced pH$_i$, TEP, and R$_T$ responses in the presence of apical amiloride.

In four experiments, amiloride did not affect the 13% apical CO$_2$-induced acidification ($\Delta$pH$_i = 0.23 \pm 0.01$) compared to control ($\Delta$pH$_i = 0.22 \pm 0.02$; p > 0.05). These experiments indicate that 13% apical CO$_2$-induced acidification did not activate NHE, therefore it could not have contributed to the CO$_2$-induced [Na]$_i$ increase. This lack of participation might have occurred for three reasons: (1) the 13% CO$_2$-induced acidification was too small; (2) there was no change in the proton-gradient across the Na/H exchanger; (3) the 13% CO$_2$ equilibrated Ringer is acidic relative to control (pH 7.09 vs. 7.5) and the low extracellular pH may have inhibited the Na/H exchanger (Aronson et al., 1983). We ruled out the first possibility with a 10 mM NH$_4$ pre-pulse.
that caused only $\approx 0.1$ decrease in $pH_i$ ($n = 4$) but still showed the characteristic Na/H exchanger mediated $pH_i$ recovery; in comparison, 13% apical CO$_2$ acidified the cell by more than 0.2 pH-units. In addition, we showed that reducing basal bath [HCO$_3$] acidified the cell by only $\approx 0.2$ but was able to activate the Na/H exchanger. Na/2HCO$_3$ entry via NBC1 was eliminated as a possible cause of the 13% apical CO$_2$-induced $[Na]_i$-increase because: (1) the apical DIDS induced $pH_i$ and TEP responses were the same in 5% or 13% apical bath CO$_2$ (Fig. 3-9); (2) the magnitude of the 13% apical CO$_2$-induced $pH_i$ response was unaltered in the presence of apical DIDS (Fig. 3-11).

In alveolar epithelium, 3Na/2K ATPase activity is reduced by CO$_2$-induced acidification (Briva et al., 2007), suggesting the possibility of a similar effect in RPE. In frog RPE, 13% CO$_2$-induced acidification activated the Na/K/2Cl co-transporter (NKCC1) due to a reduction in $[Cl]_i$ that follows the inhibition of the basolateral membrane Cl/HCO$_3$ exchanger (Edelman et al., 1994). Both the 13% apical CO$_2$-induced inhibition of the 3Na/2K ATPase and activation of the Na/K/2Cl co-transporter can increase $[Na]_i$. The increased $[Na]_i$ should facilitate NaHCO$_3$ transport via the basolateral membrane NBC. However, NBC only requires one Na$^+$ ion for every three HCO$_3^-$ ion transported, thus Na may not be the limiting substrate for this Na/3HCO$_3$ co-transporter.
Section 3.7 – Basolateral membrane NBC: dependence on Na or HCO₃ as substrate

Since [Na]ᵢ is low (15 mM) compared to [Na]ₒ (≈ 140 mM), we expected Na-linked transporters at the apical membrane (Fig. 1-3) to provide substrate that would help drive the outward transport of Na/nHCO₃ at the basolateral membrane. There are four major Na-linked transporters at the apical membrane: (1) Na/H exchanger (NHE); (2) Na/K/2Cl co-transporter (NKCC1); (3) 3Na/2K ATPase (ATP); (4) Na/2HCO₃ co-transporter (NBC1). If NHE (Na⁺ in; H⁺ out) provides the main source of [Na]ᵢ for basolateral membrane Na/nHCO₃ transport out of the cell, inhibition of NHE with amiloride should reduce basolateral membrane Na/nHCO₃ activity.

Fig. 3-24: Basal bath Δ[HCO₃⁻]-induced pHᵢ, TEP, and Rᵢ responses in the presence of apical amiloride.
To test this notion, we reduced basal bath [HCO₃⁻] (10-fold) and observed the resultant pHᵢ and TEP responses in the presence or absence of 1 mM apical amiloride (Fig. 3-24). In five experiments, amiloride did not affect basal bath Δ[HCO₃⁻]-induced TEP response (Table 3-2), indicating that the apical membrane Na/H exchanger does not provide substrate for basolateral Na/nHCO₃ co-transport activity. On the other hand, the basal bath Δ[HCO₃⁻]-induced acidification and H⁺-flux was larger in the presence of apical amiloride compared to control (Table 3-1). This observation indicates that the Na/H exchanger normally acts to buffer cell acidification produced by HCO₃⁻-efflux from the basolateral membrane.

Fig. 3-25: Basal bath Δ[HCO₃⁻]-induced pHᵢ, TEP, and Rᵢ responses in the presence apical bumetanide.
Next, we test if Na-entry via NKCC1 facilitates basolateral Na/nHCO₃ co-transport activity (Fig. 3-25). We inhibited NKCC1 with 200 μM apical bumetanide, which did not affect the basal bath Δ[HCO₃⁻]-induced pHᵢ (ΔpHᵢ and H⁺-flux) and TEP responses (Tables 3-1 & 3-2). This lack of effect suggests that Na-entry via the NKCC1 does not contribute significantly to basolateral Na/nHCO₃ co-transport activity. We also evaluated the effect of Na-extrusion by the apical membrane 3Na/2K ATPase on the activity of the basolateral membrane Na/nHCO₃ co-transporter (Fig. 3-26).

![Graph](image)

**Fig. 3-26:** Basal bath Δ[HCO₃⁻]-induced pHᵢ, TEP, and Rᵢ responses in the presence apical ouabain.

Unlike NHE, NKCC1, or NBC1, the 3Na/2K ATPase transports three Na⁺ ions out of the cell in exchange for two K⁺ ions. Thus its inhibition by ouabain should increase [Na]ᵢ,
which may stimulate basolateral Na/nHCO₃ co-transport activity. Adding 200 µM ouabain into the apical bath caused an acute TEP-decrease ($\Delta$TEP = 0.55 ± 0.47 mV; n = 5), as expected from inhibition of the 3Na/2K ATPase. However, apical ouabain did not affect the basal bath $\Delta$[HCO₃⁻]-induced pHᵢ (ΔpHᵢ and H⁺-flux) and TEP responses (Tables 3-1 & 3-2), indicating that Na-extrusion by the 3Na/2K ATPase does not reduce or limit basolateral Na/nHCO₃ co-transport activity.

The basolateral membrane Na/nHCO₃ co-transporter may be dependent on Na and HCO₃ entry from the apical membrane via the electrogenic Na/2HCO₃ co-transporter (NBC1). Therefore, we tested the coupling between the apical and basolateral membrane Na/HCO₃ co-transporters by decreasing basal bath [HCO₃⁻] (10-fold) in the presence of 0.5 mM 0.5 mM DIDS (Ap)

Fig. 3-27: Basal bath $\Delta$[HCO₃⁻]-induced pHᵢ, TEP, and $R_T$ responses in the presence apical DIDS.
apical DIDS (Fig. 3-27). In seven experiments, the basal bath $\Delta [\text{HCO}_3^-]$-induced TEP response decreased from $0.86 \pm 0.17$ to $0.49 \pm 0.06$ mV ($p < 0.05$) in the presence of apical DIDS, suggesting that inhibiting apical HCO$_3^-$-entry via the Na/2HCO$_3^-$ co-transporter reduces the HCO$_3^-$-supply that drives basolateral Na/nHCO$_3^-$ co-transport. However, apical DIDS increased the basal bath $\Delta [\text{HCO}_3^-]$-induced acidification and H$^+$-flux by $\approx 20\%$ (Table 3-1). This observation suggests that normally, apical HCO$_3^-$-entry via the Na/2HCO$_3^-$ co-transporter is a buffer that counteracts the acidification caused by HCO$_3^-$-efflux from the basolateral membrane.

From these experiments, we conclude that HCO$_3^-$ (not Na) is the limiting substrate for the basolateral membrane Na/nHCO$_3^-$ co-transporter. This is supported by the following evidence: (1) reducing basal bath [HCO$_3^-$] 10-fold caused a TEP response that was reduced in the presence of apical DIDS, suggesting that inhibiting NBC1 reduced basolateral Na/nHCO$_3^-$ co-transport activity; (2) the basal bath $\Delta [\text{HCO}_3^-]$-induced TEP response was reduced in the presence of basal DZA, suggesting that CA-inhibition reduces basolateral Na/nHCO$_3^-$ co-transport. DZA reduces HCO$_3^-$-transport in two ways: first, DZA slows CA-mediated hydration of CO$_2$ to HCO$_3^-$; second, DZA inhibits the apical membrane Na/2HCO$_3^-$ co-transporter, as evidenced by the reduction of apical bath $\Delta [\text{HCO}_3^-]$-induced TEP response in the presence of apical DZA; (3) the basal bath $\Delta [\text{HCO}_3^-]$-induced TEP response was increased in 13% apical bath CO$_2$ and decreased in 1% apical bath CO$_2$. This suggests that apical CO$_2$-entry and its subsequent conversion into HCO$_3^-$ is an important source of HCO$_3^-$-substrate for basolateral Na/nHCO$_3^-$ co-transport activity.
In addition to showing that the basolateral membrane Na/nHCO$_3$ co-transporter is dependent on apical HCO$_3$-supply, we also eliminated Na as a limiting substrate for basolateral Na/nHCO$_3$ co-transport by examining three Na-transport proteins at the apical membrane of the RPE (Hughes et al., 1998): (1) amiloride-sensitive Na/H exchanger (NHE); (2) bumetanide-sensitive Na/K/2Cl co-transporter (NKCC1); (3) ouabain-sensitive 3Na/2K ATPase (ATP). In hfRPE, the presence of amiloride, bumetanide, or ouabain in the apical bath had no effect on the basal bath $\Delta$[HCO$_3$]-induced TEP responses, suggesting that these Na-transport mechanisms are not linked to basolateral Na/nHCO$_3$ co-transport activity. Taken together, our data indicate that the basolateral membrane Na/nHCO$_3$ co-transporter is mainly driven by HCO$_3$ supplied by NBC1 mediated Na/2HCO$_3$ entry and CA II-mediated hydrolysis of CO$_2$ to HCO$_3$. 
Sections 3.5 & 3.6 conclusions: Increasing apical CO₂ increases intracellular Na⁺.

HCO₃⁻-transport via basolateral membrane NBC is dependent on HCO₃⁻-transport via NBC1.

**Supporting experimental observations**

- 13% apical CO₂ increases [Na⁺]
- Apical amiloride did not affect steady-state pH at 5% or 13% apical CO₂
- Apical amiloride did not affect 13% apical CO₂ induced acidification
- Basal bath Δ[HC0₃⁻] induced TEP response is unaffected by apical amiloride
- Basal bath Δ[HC0₃⁻] induced TEP response is unaffected by apical ouabain
- Basal bath Δ[HC0₃⁻] induced TEP response is reduced by apical DIDS

**Interpretation**

- Increasing apical bath CO₂ activates Na⁺-dependent mechanisms at the apical membrane
- Increasing apical bath CO₂ did not activate NHE-mediated Na⁺ exchange
- Increasing apical bath CO₂ did not activate NHE-mediated Na⁺ exchange
- Electrically HCO₃⁻-transport at the basolateral membrane is DIDS-sensitive
- HCO₃⁻ is transported at the basolateral membrane via both AE2 and NBC
- Electrically HCO₃⁻-transport at the basolateral membrane is Na⁺-dependent
- HCO₃⁻-transport via basolateral membrane NBC is dependent on NBC1 activity

**Fig.**

- 3:21
- 3:22
- 3:23
- 3:24
- 3:25
- 3:26
- 3:27
Section 3.8 – CO₂ induced fluid transport in hfRPE

Fig. 3-28: CO₂ induced ion and fluid transport in human retinal pigment epithelium. CO₂ enters the RPE at its apical membrane by diffusion, or it may be transported in the form of HCO₃⁻ via NBC1 and NBC3. CO₂ exits RPE basolateral membrane as HCO₃⁻, mainly via basolateral membrane NBC – this process is facilitated by carbonic anhydrase II (CA II) activity.

The model in Fig. 3-28 predicts that 13% apical CO₂ would increase net Na, Cl, and HCO₃⁻ absorption, producing an increase in steady-state fluid-absorption (Jᵥ) across the RPE. In four experiments, increasing CO₂ from 5% to 13% in both solution baths increased Jᵥ by more than 2-fold (from 2.8 ± 1.6 to 6.7 ± 2.3 µl·cm⁻²·hr⁻¹; n = 5; p < 0.05). In another set of experiments, decreasing CO₂ from 5% to 1% in both solution baths decreased steady-state fluid absorption by ≈ 60% (from 8.8 ± 3.9 to 3.4 ± 1.1 µl·cm⁻²·hr⁻¹; n = 4; p < 0.05).
In bovine RPE, net active Cl absorption is mediated by the Na/K/2Cl co-transporter at the apical membrane (Edelman et al., 1994) and by Ca\(^{2+}\)-activated and cAMP/PKA-dependent CFTR Cl channels at the basolateral membrane (Joseph & Miller, 1991; Bialek et al., 1995; Hughes et al., 1998). Evidence for the expression and basolateral membrane localization of CFTR in hfRPE has been presented (Blaug et al., 2003). The 13% apical CO\(_2\)-induced activation of the Na/K/2Cl co-transporter and inhibition of the Cl/HCO\(_3\) exchanger would both increase net Cl absorption across the RPE.

HCO\(_3\) transport also plays a significant role in RPE fluid transport. We tested the role of the apical membrane Na/2HCO\(_3\) co-transporter in fluid transport by adding 0.5 mM DIDS into the apical bath and measuring the resultant change in steady-state fluid absorption rate \(J_V\). In four experiments, 0.5 mM apical DIDS decreased \(J_V\) by more than 50% (from 16.7 ± 4.8 to 7.7 ± 3.7 µl·cm\(^{-2}\)·hr\(^{-1}\); \(p < 0.05\)), suggesting that HCO\(_3\)-transport via NBC1 mediates a major component of fluid absorption across the RPE apical membrane. The presence of NBC3 at the apical membrane suggests that it also contributes to HCO\(_3\)-mediated fluid transport. At the basolateral membrane, HCO\(_3\) transporters, and several Cl-channels are DIDS-sensitive. Not surprisingly, the addition of 0.5 mM DIDS to the basal bath decreased \(J_V\) from 20.3 ± 8.2 to 11.2 ± 6.0 µl·cm\(^{-2}\)·hr\(^{-1}\) \((n = 9; p < 0.05)\). In addition, dorzolamide or acetazolamide decreases steady-state fluid absorption across hfRPE *in vitro* (Zhi et al., ARVO 2007, #2532). These observations are corroborated in the present experiments by the DZA-induced inhibition of NBC1 the apical membrane and the Na/nHCO\(_3\) co-transporter at the basolateral membrane.
Interestingly, animal models showed that systemically administered acetazolamide increases fluid absorption across the RPE (Wolfensberger, 1999). Furthermore, clinical studies showed that acetazolamide reduces macular edema in some patients with retinitis pigmentosa and uveitis (Cox et al., 1988; Fishman et al., 1989). It has been proposed that acetazolamide increases RPE fluid absorption \textit{in vivo} by affecting membrane-bound carbonic anhydrases at the basolateral membrane (Wolfensberger, 1999), but there is no known membrane-bound CAs at the basolateral membrane of native RPE (Zhi et al., ARVO 2007, #2532). In addition, acetazolamide readily permeates RPE basolateral membrane, which would reduce fluid absorption by inhibiting cytosolic CA II, as observed \textit{in vitro} (Zhi et al., ARVO 2007, #2532). More recently, Xu and colleagues show that acetazolamide induces hypoxia-inducible factor 1 alpha (HIF-1\(\alpha\)) expression in rat cerebral cortex (Xu et al., 2009). This finding suggest the possibility that chronic exposure of RPE cells to acetazolamide may exert its therapeutic effects against macular edema via HIF-1\(\alpha\)-mediated regulation of proteins involved in fluid transport. Therefore, the key to understanding why acetazolamide has opposite effects on RPE fluid transport \textit{in vitro} vs. \textit{in vivo} may lie in the difference between acute and chronic effects of acetazolamide on RPE physiology.
Section 3.9 – HCO₃-mediated ion and fluid transport in the choroid plexus epithelium—a comparison with retinal pigment epithelium

Both the RPE and the choroid plexus epithelium (CPE) develop from neural ectoderm, therefore it is not surprising to find many similarities in the solute transport mechanisms of these two epithelia (Hughes et al., 1998; Brown et al., 2004; Praetorius, 2007). As demonstrated in this study, HCO₃-transport mediates net solute and fluid absorption in human RPE. This is supported by experiments where acetazolamide or dorzolamide reduced steady-state fluid absorption by \( \approx 50\% \) in hfRPE cultures (Zhi et al., ARVO 2007, #2532). Similarly in the CPE, HCO₃-transport is an important mediator of CSF-production (Saito & Wright, 1983, 1984); CSF-secretion is inhibited by basal DIDS (Deng & Johanson, 1989). In addition, acetazolamide reduces CSF-secretion by \( \approx 40\% \) (Vogh et al., 1987). Acetazolamide is used to prevent cerebral edema at high altitudes (Wright et al., 2008) and to reduce CSF-pressure in children with hydrocephalus (Cowan & Whitelaw, 1991). The inhibitory effect of acetazolamide on CSF-secretion led to the notion that CO₂-entry into the CPE from the blood-plasma and the subsequent hydration of CO₂ into HCO₃ stimulates NaHCO₃-secretion across the apical membrane. This conclusion is supported by experiments in cat CPE, where \( \approx 40\% \) of Na-secretion is attributed to CA II-mediated HCO₃ formation from CO₂ (Vogh & Maren, 1975). Perhaps not surprising, this mechanism of CO₂-driven HCO₃ transport is also found in the RPE.

Despite many similarities, the RPE normally absorbs Na (Cl + HCO₃) and fluid, while the CPE secretes Na (Cl + HCO₃) and fluid which helps form cerebrospinal fluid (CSF). As in the RPE, the CPE expresses Na/HCO₃ co-transporters at both the apical and
basolateral membranes. However, the most striking difference is that both NBC1/NBCe1 and NBC3/NBCn1 in the RPE are expressed at the apical membrane (Zhi et al., ARVO 2007, #2532), whereas in the CPE, these two transporters are expressed at the basolateral membrane (Brown et al., 2004; Praetorius, 2007). This difference suggests that NBC4, which is found at the apical membrane of the CPE (Millar & Brown, 2008), may be the unidentified Na/nHCO3 co-transporter at the basolateral membrane of the RPE. We hypothesize that the difference in the membrane location of these HCO3 transporters (i.e., NBC1, NBC3, and NBC4) in the RPE and CPE is the basis for their difference in HCO3 and fluid transport direction.

If the CO2-permeability difference of the apical and basolateral membranes of the RPE also manifests in the CPE, what is its functional significance? In the central nervous system, metabolic CO2 produced by the brain is released into the CSF, and is subsequently neutralized by HCO3 secreted from the CPE. We hypothesize that the CPE has a relatively lower CO2 permeability at the apical membrane than at the basolateral membrane, and this property would promote CA II-mediated HCO3 secretion across the apical membrane. This possibility remains to be evaluated.
Section 3.10 – Physiological implications

Upon dark adaptation, oxygen consumption in the retina increases (Kimble et al., 1980; Medrano & Fox, 1995; Cringle et al., 2002; Yu & Cringle, 2002), thus generating and depositing more CO₂ and H₂O into the SRS. Both CO₂ and H₂O generation can be estimated from the rate of oxygen consumption measured *in situ* in cat and non-human primate eyes (Wangsa-Wirawan & Linsenmeier, 2003). Our calculations (section 3-11) provide an estimate of CO₂ production in adult human photoreceptors of ≈ 0.29 and 0.54 mmol·hr⁻¹ in light and dark respectively. Considering that SRS [CO₂] is ≈ 2 mM, impaired CO₂-transport across the RPE could cause significant SRS or RPE acidification resulting in photoreceptor or RPE cell death. In addition, oxidative phosphorylation in the adult retina produces water at a rate of ≈ 0.5 µl·cm⁻²·hr⁻¹ in light and 0.9 µl·cm⁻²·hr⁻¹ in dark adapted eyes. Since glycolysis in the retina accounts for ≈ 95% of its total glucose consumption (Winkler et al., 2008), the combined retinal water production by aerobic respiration and glycolysis is calculated to be 3.6 and 6.5 µl·cm⁻²·hr⁻¹ in the light and dark respectively. The CO₂ induced changes in ion-transport in the RPE is one of many events that follows the transition from light to dark *in vivo*, others include: (1) an increase in SRS [K⁺] from ≈ 3 to 5 mM; (2) the decrease in SRS [Ca²⁺]; (3) the decrease in SRS pH (Steinberg et al., 1983; Borgula et al., 1989; Livsey et al., 1990; Yamamoto et al., 1992; Gallemore et al., 1994). Dark-adaptation decreases SRS volume in situ (Li et al., 1994a; Li et al., 1994b). In addition, in a rat model of retinal re-attachment (Maminishkis et al., 2002), fluid clearance from the SRS was faster in the dark-adapted eye (Maminishkis A., personal communication), suggesting that steady-state fluid-absorption across the RPE is higher in the dark.
In the dark adapted eye, the high oxidative metabolism in the inner segments of the photoreceptors generates CO$_2$ and H$_2$O that are deposited into the SRS. The RPE utilizes the limited CO$_2$-diffusion at the basolateral membrane to drive Na, Cl, and HCO$_3$ transport across the RPE, which increases solute-driven fluid-transport – this mechanism not only prevents CO$_2$ accumulation in the SRS, but also removes water from the vicinity of the photoreceptors. This helps maintain the proper anatomic relationship between the photoreceptors and the RPE apical membrane, thus avoiding retinal-detachment and photoreceptor degeneration (Stone et al., 1999; Wickham et al., 2006; Nakazawa et al., 2007).
Relative CO₂ membrane permeability

\[
\frac{d[CO_2]}{dt} = D \cdot (CO_{2,in} - CO_{2,out})
\]

Differentiating the CO₂/HCO₃ equilibrium constant,

\[
\frac{d[CO_2]}{dt} = \frac{[HCO_3^-]}{K_a} \cdot \frac{d[H^+]}{dt}
\]

Combining the above equations give,

\[
\frac{[HCO_3^-]_{in}}{K_a} \cdot \frac{d[H^+]}{dt}_{Ap} = D_{Ap} \cdot (CO_{2,in} - CO_{2,out})
\]

\[
\frac{[HCO_3^-]_{in}}{K_a} \cdot \frac{d[H^+]}{dt}_{Ba} = D_{Ba} \cdot (CO_{2,in} - CO_{2,out})
\]

The relative permeability \( P \) of CO₂ at the apical vs. the basolateral membrane is,

\[
P = \frac{D_{Apical}}{D_{Basal}} = \frac{\frac{d[H^+]}{dt}_{Ap}}{d[H^+]}_{Ba} + \frac{d[H^+]}{dt}_{Ba}
\]

Where \( D \) is the diffusion coefficient and \( \frac{d[H^+]}{dt}_{Ap} \) and \( \frac{d[H^+]}{dt}_{Ba} \) are the H⁺-fluxes caused by perfusing 13% CO₂ equilibrated Ringer to the apical and basal bath respectively. The H⁺-fluxes were obtained by multiplying the 13% CO₂ induced dpHi/dt with the total buffering capacity of the hfRPE. Based on these considerations, the relative permeability of apical vs. basolateral membrane of hfRPE to CO₂ is 9.9 ± 4.4 (n = 7).
Retinal water production by aerobic respiration

In the dark, outer retina O₂ consumption (Wangsa-Wirawan & Linsenmeier, 2003) is: 4.2 ± 0.5 ml O₂·100g⁻¹·min⁻¹

In the light, outer retina O₂ consumption (Wangsa-Wirawan & Linsenmeier, 2003) is: 2.3 ± 0.6 ml O₂·100g⁻¹·min⁻¹

Wet weight of human retina (Bhosale & Bernstein, 2005) is: 5.44 g

Oxygen consumption in the dark (density of oxygen at 36.9 °C is 0.039 mmol/ml):

\[
0.042 \text{ ml } O_2 \cdot g^{-1} \text{ min}^{-1} \times 60 \text{ min} \cdot \text{ hr}^{-1} \times 5.44 \text{ g} \times 0.0393 \text{ mM} \cdot \text{ ml}^{-1} = 0.54 \text{ mmol } O_2 \cdot \text{ hr}^{-1}
\]

Oxygen consumption in the light:

\[
0.023 \text{ ml } O_2 \cdot g^{-1} \text{ min}^{-1} \times 60 \text{ min} \cdot \text{ hr}^{-1} \times 5.44 \text{ g} \times 0.0393 \text{ mM} \cdot \text{ ml}^{-1} = 0.29 \text{ mmol } O_2 \cdot \text{ hr}^{-1}
\]

In aerobic respiration, one molecule of water is generated for every molecule of oxygen consumed. Therefore, water generated in the dark is:

\[
0.54 \text{ mmol } O_2 \cdot \text{ hr}^{-1} \times 18 \text{ mg} \cdot \text{ mmol}^{-1} \times 1 \mu l \cdot \text{ mg}^{-1} = 9.72 \mu l \text{ H}_2O \cdot \text{ hr}^{-1}
\]

Water generated in the light is:

\[
0.29 \text{ mmol } H_2O \cdot \text{ hr}^{-1} \times 18 \text{ mg} \cdot \text{ mmol}^{-1} \times 1 \mu l \cdot \text{ mg}^{-1} = 5.22 \mu l \text{ H}_2O \cdot \text{ hr}^{-1}
\]

Assuming that the entire retina surface is 10.94 cm² (http://webvision.med.utah.edu/), the total rate of fluid generated by the retina through aerobic respiration:

In the dark: \[
\frac{9.72 \mu l H_2O / hr}{10.94 \text{ cm}^2} = 0.89 \mu l \text{ H}_2O \cdot \text{ cm}^{-2} \cdot \text{ hr}^{-1}
\]

In the light: \[
\frac{5.31 \mu l H_2O / hr}{10.94 \text{ cm}^2} = 0.48 \mu l \text{ H}_2O \cdot \text{ cm}^{-2} \cdot \text{ hr}^{-1}
\]
Total retinal water production in the light and dark

For every glucose molecule that undergoes aerobic respiration, six molecules of CO$_2$ are produced. Therefore, glucose consumption by aerobic respiration in the dark is:

$$0.54 \text{ mmol} \cdot \text{hr}^{-1} \times \frac{1}{6} \text{ glu} \cos e = 0.09 \text{ mmol} \cdot \text{hr}^{-1}$$

Glucose consumption by aerobic respiration in the light is:

$$0.29 \text{ mmol} \cdot \text{hr}^{-1} \times \frac{1}{6} \text{ glu} \cos e = 0.05 \text{ mmol} \cdot \text{hr}^{-1}$$

Assuming that glycolysis in the retina accounts for 95% of glucose consumption in the dark (Winkler et al., 2008), the rate of water generation by glycolysis in the dark is:

$$0.09 \text{ mmol} \cdot \text{hr}^{-1} \times \frac{95}{5} \times \frac{2}{1} \text{ glu} \cos e \times \frac{18 \mu l}{1 \text{ mmol}} \times \frac{1}{10.94 \text{ cm}^2} = 5.6 \mu l \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$$

The rate of water generation by glycolysis in the light is:

$$0.05 \text{ mmol} \cdot \text{hr}^{-1} \times \frac{95}{5} \times \frac{2}{1} \text{ glu} \cos e \times \frac{18 \mu l}{1 \text{ mmol}} \times \frac{1}{10.94 \text{ cm}^2} = 3.1 \mu l \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$$

Total water produced by aerobic respiration and glycolysis in dark:

$$= 0.89 + 5.6 = 6.5 \mu l \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$$

Total water produced by aerobic respiration and glycolysis in light:

$$= 0.48 + 3.1 = 3.6 \mu l \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$$

$J_V$ of human RPE in vivo has been estimated using B-scan ultrasonography to be $\approx 11$ $\mu l \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$ (Chihara & Nao-i, 1985), comparable to our in vitro measurements (Fig. 13).
**CO₂ production in the light and dark**

CO₂ production = O₂ consumption. CO₂ production in dark: 4.2 ± 0.5 ml CO₂·100g⁻¹ min⁻¹, and in light: 2.3 ± 0.6 ml O₂·100g⁻¹ min⁻¹. Therefore, CO₂ production increases by 1.4 – 2.6 fold after transitioning from light to dark. This increase in CO₂ production translates to an increase in SRS CO₂ concentration, from 5% to 10 ± 3%.
CHAPTER 4: Lactate Induced Ion Transport Mechanisms in RPE

Section 4.1 – Introduction

Photoreceptor metabolism is higher in the dark than in light (Yamamoto et al., 1992; Wang et al., 1997a; Wang et al., 1997b; Padnick-Silver & Linsenmeier, 2002; Winkler et al., 2008) and it is estimated that ATP consumption by rod photoreceptor is ≈ 4-fold higher in the dark (Okawa et al., 2008). The high ATP production is needed to drive the Na/K ATPase and Ca^{2+} ATPase at the photoreceptor inner segments, to maintain the “dark current” that circulates between the photoreceptor inner and outer segments (Ames et al., 1992; Krizaj & Copenhagen, 1998; Okawa et al., 2008). Therefore in the dark, oxidative metabolism at the photoreceptor inner segments increases, thus reducing the local oxygen levels (Wangsawan & Linsenmeier, 2003) and increasing the need for glycolysis-derived ATP. More than 80% of all glucose consumed by photoreceptors is converted to lactic acid (Wang et al., 1997a; Wang et al., 1997b; Winkler et al., 2008) indicating that the retina is highly dependent on glycolysis as a source of ATP, even in the presence of oxygen (Winkler et al., 2000; Padnick-Silver & Linsenmeier, 2002; Winkler et al., 2004). This finding is consistent with the high lactate concentration (4 - 13 mM) in the subretinal space (SRS) compared to that in blood (≈ 1 mM) (Adler & Southwick, 1992). Although lactate released by Müller cells in darkness can be used as an energy source for photoreceptor activity (Poitry-Yamate et al., 1995; Poitry et al., 2000), this mechanism is controversial (Winkler et al., 2004). Regardless of the source, more lactic acid are generated in the dark and released from the retina into the SRS.
The choroidal circulation (see chapter 1) is the main pathway for the removal of photoreceptor-generated lactic acid (and CO₂) from the SRS. This process is mediated by the RPE, which expresses proton-coupled lactate (H⁺/Lac⁻) transporters of the MCT family at its apical (MCT1) and basolateral membranes (MCT3) (Kenyon et al., 1994; la Cour et al., 1994; Lin et al., 1994; Zeuthen et al., 1996; Philp et al., 1998; Hamann et al., 2003; Philp et al., 2003a; Philp et al., 2003b; Majumdar et al., 2005). MCT lactate-transporters are electroneutral, but early in vitro experiments in bovine RPE showed that adding lactate to the apical bath alters RPE membrane voltage, suggesting involvement of electrogenic mechanisms (Kenyon et al., 1994). In this study, we investigate these lactate-activated mechanisms. We demonstrate that apical lactate entry stimulates KCl-efflux via a Ba²⁺-sensitive K-channel at the apical membrane and ClC-2 Cl-channel at the basolateral membrane. These channels may be activated by lactate-induced cell-swelling (Zeuthen et al., 1996; Hamann et al., 2003), but we show that activation of these two channels is caused by cell acidification. Lactate-induced KCl-efflux decreases RPE cell volume and prevent swell-induced osmotic stress. In addition, lactate-induced activation of apical membrane K-channel may be an important mechanism that regulates SRS K-homeostasis, which is critical for photoreceptor/RPE interactions in light-dark transitions.

All experiments presented in this chapter are performed in CO₂/HCO₃-free condition because the lactate-induced responses are more pronounced and can be easily measured. Although this eliminates interactions between lactate and HCO₃ transport mechanisms, the main features of lactate-induced pHᵢ, TEP, and Rₜ responses were preserved (Fig. 5-1). Interactions between lactate and HCO₃-transporters are explored in chapter 5.
Section 4.2 – MCT localization in cultured hfRPE

Studies in mouse and human RPE showed MCT1 and MCT3 localization at the apical and basolateral membranes, respectively (Philp et al., 1998; Philp et al., 2003b). First, we confirm the localization of these MCTs in hfRPE cultures since this model will be used for all physiological experiments. Western blots showed that MCT1, MCT3, and MCT4 and their accessory protein (CD147) are expressed in cultured hfRPE cells (Fig. 4-1A). Immunofluorescence imaging shows that MCT1 is localized at the apical membrane, whereas both MCT3 and MCT4 are localized to the basolateral membrane (Fig. 4-1B). CD147 was detected in both apical and basolateral membranes; CD147 labeling of the basolateral membrane is weak probably due to epitope masking by MCT 3 or 4. CD147 (or basigin) is an accessory protein that associates with MCTs to regulate their membrane targeting and localization – its detection indicates proper distribution of MCTs in cultured hfRPE.

Fig. 4-1: Expression and localization of CD147 and MCTs in cultured hfRPE monolayer. (A) Western blot (CG – core-glycosylated, 29.2 kD; FG – fully-glycosylated, 59 kD); (B) immunolabeling.
MCT4 is normally absent in native RPE, but it is expressed in cultured RPE (e.g., ARPE-19) (Philp et al., 2003b). MCT4 is expressed in highly glycolytic cells (Dimmer et al., 2000). Studies in renal cells show that MCT4 expression is upregulated in hypoxia (low O$_2$) via hypoxia-inducible factor 1-alpha (HIF-1$\alpha$) (Ullah et al., 2006). HIF-1$\alpha$ controls expression of many genes involved in glycolysis, such as lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1) (Gleadle & Ratcliffe, 1998; Semenza, 2003). When cells are placed in culture, they abandon oxidative metabolism and depend on glycolysis as the main source of energy (Gstraunthaler et al., 1999). This is because O$_2$ supply from the environment to the confluent hfRPE is limited by a large diffusion layer (4.4 mm of stagnant media), thus causing hypoxia (Pettersen et al., 2005). Based upon these studies, we infer that cultured RPE cells express high levels of MCT4 due to hypoxia-induced activation of HIF-1$\alpha$.

MCT3 ($K_m \approx 6$ mM (Grollman et al., 2000)) has significantly higher affinity for L-lactate than MCT4 ($K_m \approx 30$ mM (Philp et al., 1998)). A simple calculation with Michaelis-Menten kinetics show that MCT3 transports lactate at a rate $\approx 2$ – $4$ folds faster than MCT4 (assuming [Lactate], in RPE ranges between 2 and 20 mM). Does this mean that MCT3 (rather than MCT4) is the major H/Lac co-transporter at the basolateral membrane? Not necessarily. Although MCT3 transports lactate faster than MCT4, MCT4 may be expressed at a much higher levels than MCT3, thus compensating for its lower lactate binding affinity. Therefore, at least in cultured hfRPE, MCT4 can contribute significantly to lactate-transport.
Section 4.3 – Lactate induced pH_{i} responses in RPE

Lactate transport at the apical membrane can be studied by imposing a lactate gradient across the apical membrane. This maneuver drives lactic acid into the RPE via MCT1, thus causing intracellular acidification. In this study, we stimulate MCT1 by perfusing 20 mM lactate Ringer into the apical bath and MCT1 activity is reflected by the subsequent changes in pH_{i}, TEP, and R_{T}. As shown in Fig. 4-2, apical lactate produced a pH_{i} response with two successive phases: a fast acidification (R1) followed by a slow alkalinization (R2). In the absence of CO_{2}/HCO_{3}, we took the intrinsic buffering capacity of the cell (β_{i}) into account to determine the rate of H^{+}-entry (ΔpH_{i}/Δt x β_{i}).

![Figure 4-2: Apical monocarboxylate (i.e., lactate, pyruvate, acetate, or propionate) induced pH_{i}, TEP, and R_{T} responses.](image-url)
During R1, the hfRPE acidified at a rate of $-6.32 \pm 1.46 \text{ mM} \cdot \text{min}^{-1}$ and R2 has a rate of $0.71 \pm 0.31 \text{ mM} \cdot \text{min}^{-1}$ (n = 65). Concomitantly, apical lactate also increased TEP and $R_T$ by $1.39 \pm 0.60 \text{ mV}$ and $52 \pm 21 \Omega \cdot \text{cm}^2$ (n = 87), respectively. The pH$_i$ responses are presented in H$^+$-flux, which is determined by taking into account the intrinsic buffering capacity of the RPE. To make sure that these responses are down-stream effects of H$^+$-coupled monocarboxylate transporter (MCT1) activity, we compared the pH$_i$, TEP, and $R_T$ responses induced by apical addition of various monocarboxylates (20 mM of lactate, pyruvate, acetate, or propionate) (Fig. 4-2). Adding various monocarboxylates (i.e., pyruvate, acetate, or propionate) to the apical bath produced similar but generally larger pH$_i$, TEP, and $R_T$ responses compared to that caused by lactate (Table 4-1), suggesting that MCT1 has lower affinity for lactate than for other monocarboxylates. Based on R1 values, MCT1 substrate specificity in hfRPE (lactate = acetate $<$ pyruvate $=$ propionate) resembles MCT1 in Ehrlich Lettré tumor cells (Jackson & Halestrap, 1996). Some discrepancies can be expected due to H$^+$-coupled monocarboxylate transport at the basolateral membrane via MCT3, which also exhibits substrate preference.

Table 4-1. monocarboxylate-induced pH$_i$, TEP, and $R_T$ responses.

<table>
<thead>
<tr>
<th></th>
<th>lactate</th>
<th>pyruvate</th>
<th>acetate</th>
<th>propionate</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 (mM·min$^{-1}$)</td>
<td>-6.17 ± 0.93</td>
<td>-8.28 ± 1.85</td>
<td>-7.15 ± 1.20</td>
<td>-8.48 ± 2.51</td>
</tr>
<tr>
<td>R2 (mM·min$^{-1}$)</td>
<td>0.71 ± 0.12</td>
<td>0.87 ± 0.27</td>
<td>0.85 ± 0.23</td>
<td>0.93 ± 0.19</td>
</tr>
<tr>
<td>$\Delta$TEP (mV)</td>
<td>1.75 ± 0.45</td>
<td>1.99 ± 0.46</td>
<td>2.60 ± 0.52</td>
<td>2.70 ± 0.52</td>
</tr>
<tr>
<td>$\Delta R_T$ (Ω·cm$^2$)</td>
<td>81 ± 15</td>
<td>77 ± 10</td>
<td>66 ± 12</td>
<td>75 ± 12</td>
</tr>
</tbody>
</table>

a. values are presented as mean ± SD from five tissues.

To test the activity of MCT1, we measured lactate-induced pH$_i$, TEP, and $R_T$ responses in the presence of MCT1 inhibitors. In the first experiment, we perfused lactate Ringer to
the apical bath in the presence or absence 50 μM apical pCMBS (p-chloromercuribenzenesulfonic acid; MCT1 inhibitor) (Fig. 4-3).

Fig. 4-3: Apical lactate induced pHi, TEP, and RT responses in the presence of apical pCMBS.

Adding pCMBS (50 μM) into the apical bath acidified the cells and significantly decreased steady-state TEP and $R_T$ ($\Delta pHi = -0.15 \pm 0.01$; $\Delta TEP = -11.99 \pm 0.87$ mV; $\Delta R_T = -305 \pm 87 \, \Omega \cdot \text{cm}^2$; $n = 4$). In four experiments, pCMBS almost completely blocked the apical lactate-induced pHi, TEP, and $R_T$ responses (Table 4-2) – the effects of pCMBS on the pHi, TEP, and $R_T$ responses were irreversible even after 5 min washout.
Table 4-2. Apical lactate-induced pH<sub>i</sub>, TEP, and R<sub>T</sub> responses.

| inhibitor/condition | Apical lactate-induced pH<sub>i</sub>, TEP, and R<sub>T</sub> responses<sup>b</sup> |  |
|---------------------|------------------------------------------|---|---|---|---|
|                     | Control | w/ inhibitor | Recovery | p<sup>c</sup> | n  |
| Apical Basal        |          |              |          |               |    |
| pcMBs               |          |              |          |               |    |
| R1                  | -8.19 ± 0.94 | -1.03 ± 0.09 | -1.51 ± 0.20 | S  | 4  |
| R2                  | 1.09 ± 0.06  | 0.13 ± 0.08  | -0.06 ± 0.08 | S  |    |
| ∆TEP                | 1.99 ± 0.31  | 0.04 ± 0.08  | 0.24 ± 0.29  | S  |    |
| ∆R<sub>T</sub>      | 27 ± 13     | 0            | 0          | S  |    |
| Nifflumic acid      |          |              |          |               |    |
| R1                  | -5.93 ± 1.29 | -3.22 ± 0.79 | -6.24 ± 0.89 | S  | 7  |
| R2                  | 0.78 ± 0.25  | 0.49 ± 0.23  | 0.77 ± 0.26  | S  |    |
| ∆TEP                | 1.58 ± 0.73  | 1.08 ± 0.52  | 1.47 ± 0.79  | S  |    |
| ∆R<sub>T</sub>      | 48 ± 12      | 38 ± 13      | 36 ± 11     | NS |    |
| Amiloride            |          |              |          |               |    |
| R1                  | -5.02 ± 1.22 | -7.03 ± 2.71 | -4.86 ± 1.16 | S  | 6  |
| R2                  | 0.78 ± 0.32  | 0            | 0.60 ± 0.29  | S  |    |
| ∆TEP                | 1.48 ± 0.79  | 1.39 ± 0.84  | 1.47 ± 1.01  | NS |    |
| ∆R<sub>T</sub>      | 43 ± 16      | 38 ± 15      | 37 ± 18     | NS |    |
| Na-free              |          |              |          |               |    |
| R1                  | -7.36 ± 1.47 | -14.2 ± 2.53 | -7.33 ± 4.31 | S  | 3  |
| R2                  | 1.34 ± 0.24  | 0            | 1.17 ± 0.32  | S  |    |
| ∆TEP                | 1.86 ± 0.32  | 1.15 ± 0.16  | 2.06 ± 0.24  | NS |    |
| ∆R<sub>T</sub>      | 42 ± 3       | 48 ± 22      | 46 ± 9      | NS |    |

a. Blank cells indicate that control Ringer was perfused into the corresponding bath.
b. H<sup>+</sup>-flux in R1 and R2 has units of mM·min<sup>-1</sup>, ∆TEP has units of mV, ∆R<sub>T</sub> has units of Ω·cm<sup>2</sup>, and all values are reported as mean ± SD.
c. Student’s t-test for statistical significance between apical lactate-induced response in control vs. in the presence of inhibitor. “S” indicates statistical significance (p < 0.05), “NS” indicates statistical insignificance (p > 0.05).
In a similar experiment, we tested the effect of another MCT1 inhibitor, niflumic acid (apical bath; 100 μM), on the apical lactate-induced pH$_i$ and TEP responses (Fig. 4-4). Adding niflumic acid to the apical bath acidified the cells, increased steady-state TEP, and decreased R$_T$ ($\Delta$pH$_i = -0.12 \pm 0.03$; $\Delta$TEP = 1.17 ± 0.74 mV; $\Delta$R$_T$ = -38 ± 13 Ω·cm$^2$; $n = 7$). In the presence of niflumic acid, apical lactate-induced R1 and R2 of the pH$_i$ response were significantly reduced (Table 4-2). In addition, the lactate-induced TEP response was reduced by $\approx 30\%$ (Table 4-2). In contrast, the effect of niflumic acid on lactate-induced R$_T$ response was statistically insignificant (Table 4-2).
In RPE, a Na/H exchanger (NHE) is expressed at the apical membrane and is stimulated by cell acidification (Lin et al., 1992; Kenyon et al., 1997). Therefore, R2 of the pHi response observed in Fig. 4-2, -3, -4 may reflect lactate-induced NHE activation. To test this possibility, we compared lactate induced pHi, TEP, and R_T responses in the presence of apical amiloride (1 mM; NHE inhibitor) (Fig. 4-5). In these experiments, adding amiloride per se acidified the cells, and increased steady-state TEP and R_T (ΔpHi = -0.13 ± 0.02; ΔTEP = 0.28 ± 0.09 mV; ΔR_T = 63 ± 41 Ω·cm²). In contrast, amiloride did not affect steady-state pHi in the presence of CO_2/HCO_3 (Fig. 3-22), indicating that NHE is more active in the absence of CO_2/HCO_3 buffering. Apical lactate acidified the cell with the same magnitude in the presence or absence of amiloride, but when intrinsic buffering

Fig. 4-5: Apical lactate induced pH_i, TEP, and R_T responses in the presence of apical amiloride.
capacity was taken into account, R1 of the lactate-induced pHᵢ response is significantly larger compared to control (Table 4-2), suggesting that apical lactate activated NHE during R1. In addition, the R2 recovery phase is completely abolished by apical amiloride (Table 4-2), but the TEP and Rₜ responses were unchanged.

Fig. 4-6: Apical lactate induced pHᵢ, TEP, and Rₜ responses in the absence of Na.

Since NHE (Na/H exchanger) is a Na-linked transporter, we can eliminate its activity by removing all Na from both apical and basal baths. Consistent with this notion, R2 was completely blocked by 1 mM apical amiloride (Fig. 4-6). Removing Na decreased steady-state pHᵢ and TEP, and increased Rₜ (ΔpHᵢ = -0.77 ± 0.06; ΔTEP = -2.82 ± 1.39 mV; ΔRₜ = 412 ± 88 Ω·cm²). Although NHE normally exchanges H⁺ for Na-entry, [Na]₀ removal generates a large outward Na-gradient that reverses NHE activity to transport H⁺
into the cell and cause cell acidification. As shown in Fig. 4-6, apical lactate-induced acidification in the absence of Na ($\Delta pHi = -0.14 \pm 0.02$) was smaller compared to control ($\Delta pHi = -0.22 \pm 0.01; p < 0.05$). However when buffering capacity is taken into account, R1 was $\approx$ two-folds larger in the absence of Na compared to control (Table 4-2). In addition, R2 of the pH response was absent in Na-free condition. These observations suggest that apical lactate activated NHE during R1 and R2.

In this section, we showed that apical lactate-induced pH response is a two-phased process composed of a fast intracellular acidification (R1) followed by a slow alkalinization (R2). R1 reflects H/Lac entry via MCT1 because it can be inhibited by niflumic acid and pCMBS, both are potent MCT1 inhibitors (Jackson & Halestrap, 1996; Morris & Felmlee, 2008). R2 reflects NHE activity because it can be eliminated by apical amiloride (1 mM) or by Na-removal from both apical and basal baths. As shown in Figs. 4-3 and 4-4, lactate-induced acidification is required for the concomitant TEP and $R_T$ responses, indicating that these responses originate from the activation of secondary mechanisms downstream of H/Lac entry. In the next section, we study the mechanisms underlying these TEP and $R_T$ responses.
Sections 4.1 & 4.2 conclusions: MCT1 mediates H/Lac co-transport across the apical membrane of RPE. H/Lac entry via MCT1 activates NHE.

Supporting experimental observations:
- MCT1 is immunolabeled to the apical membrane.
- MCT3 and MCT4 are immunolabeled to the basolateral membrane.
- Western blots detected MCT1, MCT3, and MCT4 proteins in RPE lysates.
- Apical lactate caused pH response with acidification (R1) and alkalization (R2).
- R1 is blocked by pCMBS (MCT1 inhibitor).
- R1 is inhibited by niflumic acid (MCT1 inhibitor).
- R1 is increased by amiloride.
- R2 is increased in the absence of Na⁺.

Interpretation:
- MCT1 is localized to the apical membrane. 4-1B
- MCT3 and MCT4 are localized to the basolateral membrane. 4-1B
- MCT1, MCT3, and MCT4 proteins are expressed in RPE. 4-1A
- Lactate entered the cell via MCT1 to generate R1. 4-2
- Lactate entered the cell via MCT1 to generate R2. 4-3
- Lactate entered the cell via MCT1 to generate R1. 4-4
- Apical lactate activates NHE during R1. 4-5
- Apical lactate activates NHE during R2. 4-5
- Apical lactate activates NHE during R1. 4-6
- Apical lactate activates NHE during R2. 4-6
Section 4.4 – Lactate-induced TEP and $R_T$ responses: involvement of Cl channels

Adding lactate to the apical bath increases TEP by $\approx 1.5$ mV. This increase in TEP can originate from $V_A$ hyperpolarization, $V_B$ depolarization, or both. The latter case may be caused by the activation of a Cl channel at the basolateral membrane, leading to basolateral Cl-efflux and the subsequent $V_B$ depolarization. If true, removing all Cl in both solution baths should eliminate this TEP response (Fig. 4-7). However, this maneuver cannot be performed with calomel electrodes (HgCl$_2$) because these electrodes use Cl as the conductive ion; absence of Cl would prevent the electrode from providing a reliable measurement of TEP and $R_T$. Therefore, we replaced the calomel electrodes with HgSO$_4$ electrodes (filled with sat. K$_2$SO$_4$). In this experiment, control and lactate Ringer solutions contains CaSO$_4$ (instead of CaCl$_2$) to provide SO$_4^{2-}$ as conductive ion.

Fig. 4-7: Apical lactate induced pH$_i$, TEP, and $R_T$ responses in the absence of Cl.
Table 4-3. Apical lactate-induced pH, TEP, and R_T responses.

<table>
<thead>
<tr>
<th>Inhibitor/condition</th>
<th>Apical lactate-induced pH, TEP, and R_T responses</th>
<th>p</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>w/ inhibitor</td>
<td>Recovery</td>
</tr>
<tr>
<td>Apical</td>
<td>Basal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl-free</td>
<td>Cl-free</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>-6.04 ± 1.31</td>
<td>-5.34 ± 0.67</td>
<td>-6.17 ± 0.98</td>
</tr>
<tr>
<td>R2</td>
<td>0.63 ± 0.34</td>
<td>0.59 ± 0.09</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td>∆TEP</td>
<td>1.72 ± 0.73</td>
<td>0.61 ± 0.36</td>
<td>1.47 ± 1.24</td>
</tr>
<tr>
<td>∆R_T</td>
<td>38 ± 19</td>
<td>90 ± 48</td>
<td>32 ± 12</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>Cl-free</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>-5.96 ± 1.42</td>
<td>-5.77 ± 0.85</td>
<td>-5.51 ± 0.71</td>
</tr>
<tr>
<td>R2</td>
<td>0.46 ± 0.28</td>
<td>0.68 ± 0.18</td>
<td>0.37 ± 0.20</td>
</tr>
<tr>
<td>∆TEP</td>
<td>1.05 ± 0.46</td>
<td>0.71 ± 0.28</td>
<td>1.05 ± 0.51</td>
</tr>
<tr>
<td>∆R_T</td>
<td>57 ± 17</td>
<td>56 ± 20</td>
<td>51 ± 18</td>
</tr>
<tr>
<td>CFTRinh-172</td>
<td>Cl-free</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>-6.98 ± 1.41</td>
<td>-6.78 ± 1.59</td>
<td>-8.33 ± 3.13</td>
</tr>
<tr>
<td>R2</td>
<td>0.95 ± 0.14</td>
<td>0.95 ± 0.22</td>
<td>1.10 ± 0.34</td>
</tr>
<tr>
<td>∆TEP</td>
<td>2.10 ± 0.78</td>
<td>2.06 ± 0.67</td>
<td>2.10 ± 0.65</td>
</tr>
<tr>
<td>∆R_T</td>
<td>54 ± 20</td>
<td>53 ± 17</td>
<td>53 ± 12</td>
</tr>
<tr>
<td>Forskolin</td>
<td>Cl-free</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>-7.18 ± 0.69</td>
<td>-7.98 ± 1.37</td>
<td>-8.26 ± 1.49</td>
</tr>
<tr>
<td>R2</td>
<td>0.28 ± 0.06</td>
<td>0.27 ± 0.14</td>
<td>0.42 ± 0.25</td>
</tr>
<tr>
<td>∆TEP</td>
<td>1.16 ± 0.27</td>
<td>1.20 ± 0.31</td>
<td>1.02 ± 0.35</td>
</tr>
<tr>
<td>∆R_T</td>
<td>64 ± 36</td>
<td>58 ± 38</td>
<td>66 ± 39</td>
</tr>
</tbody>
</table>

a. Blank cells indicate that control Ringer was perfused into the corresponding bath.
b. H⁺-flux in R1 and R2 has units of mM·min⁻¹, ∆TEP has units of mV, ∆R_T has units of Ω·cm², and all values are reported as mean ± SD.
c. Student’s t-test for statistical significance between apical lactate-induced response in control vs. in the presence of inhibitor. “S” indicates statistical significance (p < 0.05), “NS” indicates statistical insignificance (p > 0.05).
Removing Cl from both apical and basal baths increased steady-state pHᵢ and Rᵢ (ΔpHᵢ = 0.18 ± 0.08; ΔRᵢ = 285 ± 74 Ω·cm²; n = 5). In five experiments, both R1 and R2 of the apical lactate-induced pHᵢ responses were the same in the presence or absence of Cl, indicating that this maneuver did not impede apical H/Lac entry. However, the apical lactate-induced TEP-response was reduced by ≈ 3-fold in the absence of Cl (Table 4-3). In addition, apical lactate-induced Rᵢ response was more than two-fold larger (Table 4-3). Both observations suggest that lactate stimulates Cl-dependent mechanisms. However, Cl-removal did not completely eliminate the TEP response, suggesting involvement of other Cl-independent mechanisms.

Fig. 4-8: Apical lactate induced pHᵢ, TEP, and Rᵢ responses in the presence of apical bumetanide.
In RPE, CFTR and Ca^{2+}-dependent Cl-channels are major Cl-conductances at the basolateral membrane (Blaug et al., 2003; Li et al., 2009), and their activities are sustained by apical Cl-entry via NKCC1 at the apical membrane (see Fig. 1-8). Therefore, apical bumetanide (NKCC1 inhibitor) should reduce lactate-induced TEP response (Fig. 4-8). Consistent with this notion, the TEP response was reduced by $\approx 30\%$ in the presence of bumetanide compared to control (Table 4-3). Bumetanide had no effect on R1, but R2 was larger in the presence of bumetanide compared to control (Table 4-3), possibly due to bumetanide-induced decrease in $[\text{Na}]_i$ which facilitates NHE activity.

Fig. 4-9: Apical lactate induced pH$_i$, TEP, and R$_T$ responses in the presence of basal CFTRinh-172.
To identify the lactate-activated basolateral membrane Cl-conductance, we determined lactate-induced responses in the presence of CFTRinh-172, a specific CFTR-inhibitor (Ma et al., 2002), in the basal bath (Fig. 4-9). However, apical lactate-induced pH$_i$, TEP, and R$_T$ responses were unaffected by CFTRinh-172 (Table 4-3), suggesting that apical lactate did not stimulate CFTR. However, CFTRinh-172 *per se* had little effect on steady-state TEP, suggesting that CFTR may not be fully active. Therefore, we stimulated the cAMP-activated CFTR by adding 40 μM forskolin into the basal bath. Forskolin stimulates adenylyl cyclase activity which catalyzes production of cAMP from ATP. However, apical lactate-induced pH$_i$, TEP, and R$_T$ responses were also unaffected by forskolin (Table 4-3). These data indicate that lactate did not activate CFTR.

![Graph showing pH$_i$, TEP, and R$_T$ responses](image)

**Fig. 4-10:** Apical lactate induced pH$_i$, TEP, and R$_T$ responses in the presence of basal DIDS.
Table 4-4. Apical lactate-induced pHᵢ, TEP, and Rᵣ responses.

<table>
<thead>
<tr>
<th>inhibitor/condition</th>
<th>Apical lactate-induced pHᵢ, TEP, and Rᵣ responses&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Apical lactate-induced pHᵢ, TEP, and Rᵣ responses&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Apical lactate-induced pHᵢ, TEP, and Rᵣ responses&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Apical lactate-induced pHᵢ, TEP, and Rᵣ responses&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Apical lactate-induced pHᵢ, TEP, and Rᵣ responses&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
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<td>Recovery</td>
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<td></td>
<td>pHᵢ</td>
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<td></td>
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<tr>
<td>R1</td>
<td>$-5.02 \pm 1.81$</td>
<td>$-6.12 \pm 1.90$</td>
<td>$-7.42 \pm 3.16$</td>
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<tr>
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<td>$1.00 \pm 0.44$</td>
<td>NS</td>
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</tr>
<tr>
<td>$\Delta$TEP</td>
<td>$1.28 \pm 0.73$</td>
<td>$0.46 \pm 0.32$</td>
<td>$0.38 \pm 0.39$</td>
<td>S</td>
<td>5</td>
</tr>
<tr>
<td>$\Delta R_T$</td>
<td>$40 \pm 8$</td>
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<td>$26 \pm 5$</td>
<td>NS</td>
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</tr>
<tr>
<td>R1</td>
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<td></td>
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<td></td>
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<tr>
<td>R2</td>
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<tr>
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<tr>
<td></td>
<td>pHᵢ</td>
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<tr>
<td>R1</td>
<td>$-6.29 \pm 1.09$</td>
<td>$-7.18 \pm 1.37$</td>
<td>$-11.0 \pm 2.82$</td>
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<tr>
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<td>$0.18 \pm 0.20$</td>
<td>$0.57 \pm 0.16$</td>
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<tr>
<td>$\Delta$TEP</td>
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<td>$0.41 \pm 0.18$</td>
<td>$0.38 \pm 0.39$</td>
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<td>7</td>
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<tr>
<td>$\Delta R_T$</td>
<td>$60 \pm 25$</td>
<td>$70 \pm 25$</td>
<td>$47 \pm 31$</td>
<td>NS</td>
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</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>pHᵢ</td>
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<td></td>
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</tr>
<tr>
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<td>NS</td>
<td>4</td>
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<tr>
<td>R2</td>
<td>$0.54 \pm 0.11$</td>
<td>$0.12 \pm 0.20$</td>
<td>$0.73 \pm 0.21$</td>
<td>S</td>
<td>4</td>
</tr>
<tr>
<td>$\Delta$TEP</td>
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<td>$1.35 \pm 0.45$</td>
<td>S</td>
<td>4</td>
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<tr>
<td>$\Delta R_T$</td>
<td>$58 \pm 8$</td>
<td>$46 \pm 11$</td>
<td>$42 \pm 10$</td>
<td>NS</td>
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<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
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<td></td>
<td>pHᵢ</td>
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<tr>
<td>R1</td>
<td>$-6.65 \pm 1.49$</td>
<td>$-6.76 \pm 1.37$</td>
<td>$-7.35 \pm 1.19$</td>
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<tr>
<td>R2</td>
<td>$0.54 \pm 0.29$</td>
<td>$0.46 \pm 0.20$</td>
<td>$0.52 \pm 0.32$</td>
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<td>5</td>
</tr>
<tr>
<td>$\Delta$TEP</td>
<td>$1.27 \pm 0.30$</td>
<td>$0.73 \pm 0.12$</td>
<td>$1.26 \pm 0.28$</td>
<td>S</td>
<td>5</td>
</tr>
<tr>
<td>$\Delta R_T$</td>
<td>$53 \pm 25$</td>
<td>$83 \pm 45$</td>
<td>$56 \pm 29$</td>
<td>S</td>
<td>5</td>
</tr>
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</table>

a. Blank cells indicate that control Ringer was perfused into the corresponding bath.
b. $H^+$-flux in R1 and R2 has units of mM·min⁻¹, $\Delta$TEP has units of mV, $\Delta R_T$ has units of Ω·cm², and all values are reported as mean ± SD.
c. Student’s t-test for statistical significance between apical lactate-induced response in control vs. in the presence of inhibitor. “S” indicates statistical significance (p < 0.05), “NS” indicates statistical insignificance (p > 0.05).
Does lactate activate Ca\(^{2+}\)-activated Cl conductance? Ca\(^{2+}\)-activated Cl-channels can be blocked by DIDS, whereas CFTR is DIDS-insensitive (Bialek et al., 1995; Schultz et al., 1999; Quinn et al., 2001). Fig. 4-10 shows that basal DIDS did not affect steady-state pH\(_i\), but it significantly decreased TEP (2.58 ± 0.69 mV; n = 5) – consistent with inhibition of a Ca\(^{2+}\)-activated Cl conductance, but there was no significant change in pH\(_i\) or R\(_T\). In five experiments, basal DIDS reduced the lactate-induced TEP response by 64\% (Table 4-4), suggesting that lactate activated the DIDS-sensitive Ca\(^{2+}\)-dependent Cl conductance at the basolateral membrane.

Fig. 4-11: Apical lactate induced pH\(_i\), TEP, and R\(_T\) responses in the presence of apical BAPTA-AM.
The Ca\(^{2+}\) dependence of this Cl-channel was tested by chelating intracellular Ca\(^{2+}\) with BAPTA-AM (20 \(\mu\)M), which decreased [Ca\(^{2+}\)], by 60 ± 30 nM (\(n = 5\)). However, the lactate-induced TEP and \(R_T\) responses were identical in the presence or absence of BAPTA-AM (Table 4-4), suggesting that lactate did not activate a Ca\(^{2+}\)-dependent Cl channel and that another Cl-conductance may be involved. In this context, ClC-2 is an obvious candidate because it is an almost ubiquitously expressed Cl-channel (Thiemann \textit{et al.}, 1992) and its expression and activity in RPE has been previously demonstrated (Wills \textit{et al.}, 2000; Weng \textit{et al.}, 2002; Hartzell & Qu, 2003).

![Fig. 4-12: ClC-2 Expression and Localization in RPE. (A) Western Blot analysis for ClC-2 protein expression; (B) Immunofluorescence analysis for ClC-2 protein localization.](image)

The Western blot and immunocytochemistry data summarized in Fig. 4-12 show that ClC-2 (CLCN2) is highly expressed in hfRPE and localized to the basolateral membrane as well as intracellular compartments.
Since CLC-2 is inhibited by Zn$^{2+}$ (Hartzell & Qu, 2003), we used Zn$^{2+}$ to test CLC-2 activity (Fig. 4-13). Adding 200 μM Zn$^{2+}$ to the basal bath did not significantly affect steady-state pH$_i$, but it decreased TEP and increased R$_T$ ($\Delta$TEP = -1.24 ± 0.81 mV; $\Delta$R$_T$ = 51 ± 25 Ω·cm$^2$; n = 15), consistent with the inhibition of a basolateral membrane Cl-current. In the presence of basal Zn$^{2+}$, the apical lactate-induced TEP response was reduced by 64% (Table 4-4). Although Zn$^{2+}$ also increased the R$_T$ response, this effect was not statistically significant (n = 10; Table 4-4). R1 of the pH$_i$ response was unaffected by basal Zn$^{2+}$ and R2 was gradually eliminated (Table 4-4), indicating that Zn$^{2+}$ can enter the cell from the basal bath to reach the apical membrane, where it inhibits NHE-mediated Na/H exchange. This observation suggests that Zn$^{2+}$ entered the cell via
Zip1 and Zip2 (Zn$^{2+}$-channels; (Leung et al., 2008)) from the basolateral membrane.

Adding 200 μM Zn$^{2+}$ to the basal bath maximally inhibited ClC-2 because a higher Zn$^{2+}$ concentration (500 μM) only reduced lactate-induced TEP response by 55% (ΔTEP = 1.41 ± 0.41 vs. 0.63 ± 0.33 mV; n = 6; p < 0.05).

![Graph](image)

Fig. 4-14: Lactate-induced pH$_i$, TEP, and R$_T$ responses in the presence of apical Zn$^{2+}$.

To demonstrate that basally added Zn$^{2+}$ reduced lactate-induced TEP response by acting on basolateral membrane ClC-2, we tested the effect of apical Zn$^{2+}$ (200 μM) on lactate-induced responses (Fig. 4-14). In four experiments, adding Zn$^{2+}$ to the apical bath increased steady-state TEP and R$_T$ (ΔTEP = 0.42 ± 0.26 mV; ΔR$_T$ = 43 ± 10 Ω·cm$^2$), suggesting that Zn$^{2+}$ inhibited a channel at the apical membrane. However, the lactate-induced TEP response was larger in the presence of apical Zn$^{2+}$ compared to control
(Table 4-4), whereas the $R_T$ response was unaffected. Although $R_1$ of the $pHi$ response was unaffected by apical $Zn^{2+}$, $R_2$ of the $pHi$ response was significantly reduced (Table 4-4), indicating that apical $Zn^{2+}$ inhibited NHE activity. Since apical $Zn^{2+}$ did not reduce apical lactate-induced TEP response, this experiment confirms ClC-2 localization to the basolateral membrane.

If lactate activated ClC-2, we should be able to detect basolateral membrane depolarization by using intracellular microelectrodes to separately measure apical and basolateral membrane voltages ($V_A$ and $V_B$). Fig. 4-15 shows the apical lactate-induced changes in $V_A$, $V_B$, TEP, $R_T$, and $R_A/R_B$. Intracellular recordings show that apical lactate-
induced changes in membrane potentials ($\Delta V_A$, $\Delta V_B$) consist of two phases (P1 and P2). During P1, $V_A$ and $V_B$ rapidly depolarize with $\Delta V_B > \Delta V_A$ (TEP increase). During P2, $V_A$ and $V_B$ hyperpolarize relatively more slowly with $\Delta V_A > \Delta V_B$ (TEP increase). In 17 of 21 tissues tested, apical lactate increased $R_{A/B}$, consistent with activation of ClC-2 (which decreases $R_B$).

Addition of basal $Zn^{2+}$ hyperpolarized both $V_A$ and $V_B$, decreased steady-state TEP, and increased $R_T$ ($\Delta V_A = -4.56 \pm 3.14$ mV; $\Delta V_B = -6.17 \pm 4.23$ mV; $\Delta TEP = -1.78 \pm 1.16$ mV; $\Delta R_T = 78 \pm 34 \Omega \cdot cm^2$; $n = 5$) – consistent with inhibition of ClC-2 at the basolateral membrane. In the presence of basal $Zn^{2+}$, the lactate-induced $V_B$ depolarization during P1 was significantly reduced, whereas $V_A$ depolarization in P1 was unaffected (Table 4-5), suggesting that $Zn^{2+}$ blocked lactate-induced ClC-2 activation. In addition, both $V_A$ and $V_B$ hyperpolarization in P2 were significantly reduced by basal $Zn^{2+}$, suggesting that ClC-2 inhibition prevented activation of apical membrane K channels (see section 4.4). Furthermore, the lactate-induced increase in $R_{A/B}$ was significantly reduced (72%) by basal $Zn^{2+}$, but its effect on the $R_T$ response was statistically insignificant (Table 4-5).
Table 4-5. Apical lactate-induced $V_A$, $V_B$, TEP, and $R_T$ responses.

<table>
<thead>
<tr>
<th>inhibitor $^a$</th>
<th>Apical lactate-induced $V_A$, $V_B$, TEP and $R_T$ responses $^b$</th>
<th>p $^c$</th>
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<td></td>
<td>Apical                                           Basal</td>
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<td>$\Delta V_A$</td>
<td>$\Delta V_B$</td>
<td>$\Delta V_A$</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>P1</td>
<td>4.87 ± 1.40</td>
<td>5.54 ± 1.54</td>
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<tr>
<td></td>
<td>P2</td>
<td>-3.13 ± 1.27</td>
<td>-2.89 ± 1.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.94 ± 0.30</td>
<td>0.30 ± 0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>74 ± 28</td>
<td>80 ± 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.06 ± 0.03</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>P1</td>
<td>4.81 ± 0.97</td>
<td>5.74 ± 1.20</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>-3.08 ± 1.31</td>
<td>-3.09 ± 1.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.98 ± 0.44</td>
<td>0.48 ± 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 ± 30</td>
<td>67 ± 31</td>
</tr>
<tr>
<td></td>
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<td>0.09 ± 0.05</td>
<td>-0.10 ± 0.06</td>
</tr>
<tr>
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<td>5.16 ± 0.87</td>
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<td>49 ± 10</td>
<td>39 ± 10</td>
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<td>0.08 ± 0.03</td>
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<td>Ba$^{2+}$ Zn$^{2+}$</td>
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<td>1.01 ± 0.34</td>
<td>0.10 ± 0.06</td>
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<td>49 ± 19</td>
<td>68 ± 18</td>
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<td>0.09 ± 0.02</td>
<td>-0.02 ± 0.05</td>
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$^a$ Blank cells indicate that control Ringer was perfused into the corresponding bath.

$^b$ $V_A$, $V_B$, and TEP responses are presented in mV. $\Delta R_T$ has units of $\Omega \cdot cm^2$. All values are reported as mean ± SD.

123
c. Student’s t-test for statistical significance between apical lactate-induced response in control
vs. in the presence of inhibitor. “S” indicates statistical significance (p < 0.05), “NS” indicates
statistical insignificance (p > 0.05).
d. Apical lactate had no effect on R_A/R_B in two of the six tissues tested, statistical analysis was
performed with data from the remaining four tissues.
e. Apical lactate had no effect on R_A/R_B in one of the five tissues tested, statistical analysis was
performed with data from the remaining four tissues.

In summary, we provided evidence to show that apical lactate stimulated ClC-2 at the
basolateral membrane. In the first step, we show that apical lactate induced TEP
response involves Cl-transport: (1) the lactate-induced TEP response was reduced in the
absence of Cl (both apical and basal baths); (2) the TEP response was smaller in the
presence of apical bumetanide to inhibit apical Cl-entry via Na/K/2Cl co-transporter
(NKCC1); (3) the TEP response was reduced by basal DIDS. Cl-conductance at the RPE
basolateral membrane is mainly comprised of cAMP-activated CFTR and Ca^{2+}-
dependent Cl-channels (Hughes et al., 1998). Our data suggests that lactate did not
activate CFTR since lactate-induced TEP response was unaffected by either forskolin or
CFTR-inh172 in the basal bath. This Cl-channel is also not Ca^{2+}-dependent because
BAPTA-AM (a cell-permeable Ca^{2+}-chelator) decreased [Ca^{2+}], but did not affect
lactate-induced TEP response.

Next, we showed that lactate stimulated ClC-2 at the basolateral membrane with the
following observations: (1) apical lactate-induced V_B depolarization in P1 was smaller in
the presence of basal Zn^{2+}, whereas V_A depolarization remained unchanged; (2) the apical
lactate-induced TEP response was reduced by basal Zn^{2+}, but not by apical Zn^{2+}. In
addition, western blots indicate that ClC-2 protein is expressed in hfRPE membrane fractions. With immunofluorescence, we also confirmed ClC-2 localization at the basolateral membrane surface of confluent cultured hfRPE monolayers, although ClC-2 was also detected in intracellular compartments. Collectively, our data confirmed the presence and activity of ClC-2 at the RPE basolateral membrane, and that this channel is activated by apical lactate. However, ClC-2 is not the only electrogenic mechanism activated by apical lactate, as evidenced by the following experiments: (1) Cl-removal (both baths) did not completely eliminate the lactate-induced TEP response; (2) neither basal DIDS nor basal Zn\(^{2+}\) (200, 500, or 1000 µM) completely eliminated this TEP response; (3) lactate induced \(V_A\) and \(V_B\) depolarization in P1 phase was not completely blocked by basal Zn\(^{2+}\); (4) apical lactate-induced \(V_A\) and \(V_B\) hyperpolarization (in P2) but the activation of ClC-2 depolarizes \(V_A\) and \(V_B\) (in P1) – the latter observation does not reflect ClC-2 activity. The next section shows that apical lactate activates apical membrane K-channels.
**Section 4.3 conclusion:** Apical lactate activates CIC-2 Cl-channel at the basolateral membrane

<table>
<thead>
<tr>
<th>Supporting experimental observations</th>
<th>Interpretation</th>
<th>Fig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical lactate depolarized $V_{m}$ (in P1) and increased TEP</td>
<td>Apical lactate activates an electrogenic mechanism at the basolateral membrane</td>
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<td>Cl-removal reduced apical lactate-induced TEP response</td>
<td>Apical lactate activates a Cl-dependent mechanism</td>
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<td>CFTR-inh172 did not affect apical lactate-induced TEP response</td>
<td>Apical lactate did not activate CFTR</td>
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<td>Forskolin did not affect apical lactate-induced TEP response</td>
<td>Apical lactate did not activate CFTR</td>
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<tr>
<td>Basal DIDS reduced apical lactate-induced TEP response</td>
<td>Apical lactate activated DIDS-sensitive Cl-channel at the basolateral membrane</td>
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<tr>
<td>BAPTA did not affect apical lactate-induced TEP response</td>
<td>Apical lactate did not activate a Ca$^{2+}$-dependent Cl-channel</td>
<td>4-11</td>
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<tr>
<td>Basal Zn$^{2+}$ did not reduce apical lactate-induced TEP response</td>
<td>Apical lactate activates Zn$^{2+}$-sensitive CIC-2 at the basolateral membrane</td>
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</tr>
<tr>
<td>Apical lactate increased $R_{m}/R_{b}$, which is blocked by basal Zn$^{2+}$</td>
<td>Effect of Zn$^{2+}$ on CIC-2 is localized to the basolateral membrane</td>
<td>4-14</td>
</tr>
<tr>
<td>Apical lactate depolarizes $V_{m}$ (in P1), which is blocked by basal Zn$^{2+}$</td>
<td>Apical lactate activates CIC-2 (depolarizes $V_{m}$)</td>
<td>4-15</td>
</tr>
</tbody>
</table>
Given the high K-conductance at the apical membrane and that activation of this K-channel hyperpolarizes $V_A$ and increases TEP, we sought to determine if lactate activates this channel. To this end, we compared the lactate-induced pH_i, $V_A$, $V_B$, TEP, $R_T$, and $R_A/R_B$ responses in the presence or absence of 2 mM apical Ba\textsuperscript{2+} (K-channel inhibitor) (Fig. 4-16 and 4-17).

Fig. 4-16: Apical lactate induced pH\textsubscript{i}, TEP, and $R_T$ responses in the presence of apical Ba\textsuperscript{2+}.

Apical Ba\textsuperscript{2+} did not affect steady-state pH\textsubscript{i}, but it significantly depolarized $V_A$ and $V_B$, increased $R_A/R_B$, decreased TEP, and increased $R_T$ ($\Delta V_A = 12.58 \pm 4.39$ mV; $\Delta V_B = 9.74 \pm 3.36$ mV; $\Delta R_A/R_B = 0.39 \pm 0.18$; $n = 6$) ($\Delta$TEP = $-3.52 \pm 1.87$ mV; $\Delta R_T = 88 \pm 70$ $\Omega\cdot$cm\textsuperscript{2}; $n = 16$) – consistent with inhibition of apical membrane K-channels.
In five experiments, apical lactate-induced pH$_i$-response (R1 & R2) was unaffected by Ba$^{2+}$ (Table 4-4). On the other hand, lactate-induced $V_A$ depolarization in P1 was larger in the presence of Ba$^{2+}$, whereas the $V_B$ depolarization was unaffected (Table 4-5). In addition, $V_A$ and $V_B$ hyperpolarization in P2 were significantly reduced by apical Ba$^{2+}$. Further, the lactate-induced TEP response was reduced by almost 2-fold (from $\Delta$TEP = 1.11 ± 0.39 mV to 0.59 ± 0.17 mV; n = 11; p < 0.05), and the $R_T$ response was increased by $\approx$ 30% (from $\Delta R_T = 57 \pm 27$ to $74 \pm 37$ $\Omega\cdot$cm$^2$; n = 11; p < 0.05). These observations are consistent with activation of Ba$^{2+}$-sensitive K-channels at the apical membrane.
If apical lactate stimulated Ba$^{2+}$-sensitive K-channels and Zn$^{2+}$-sensitive ClC-2 at the apical and basolateral membrane respectively, adding apical Ba$^{2+}$ and basal Zn$^{2+}$ simultaneously should eliminate the lactate-induced TEP response (Fig. 4-18). In these experiments, apical lactate-induced responses were measured first in the presence of basal Zn$^{2+}$, followed by both apical Ba$^{2+}$ and basal Zn$^{2+}$. In the presence of basal Zn$^{2+}$, the lactate-induced TEP response was reduced from $1.02 \pm 0.33$ to $0.51 \pm 0.17$ mV ($n = 6; \ p < 0.05$). This TEP response was further decreased from $0.51 \pm 0.17$ to $0.22 \pm 0.13$ mV ($n = 6; \ p < 0.05$) in the presence of both apical Ba$^{2+}$ and basal Zn$^{2+}$. In contrast, the
effect of Ba\(^{2+}\) and Zn\(^{2+}\) on the RT response was statistically insignificant. The incomplete inhibition of the TEP response and the lack of effect on RT suggest the involvement of additional mechanisms – one that increases both TEP and RT. A possible mechanism is lactate-induced inhibition of a basolateral membrane K-channel. If lactate-induced inhibition of basolateral membrane K-channel contributed to the initial \(V_A\) and \(V_B\) depolarization in P1, basal Ba\(^{2+}\) (2 mM) should reduce this response (Fig. 4-19).

Fig. 4-19: Apical lactate induced \(V_A\), \(V_B\), TEP, \(R_A/R_B\), RT responses in the presence of basal Ba\(^{2+}\).

Adding Ba\(^{2+}\) to the basal bath depolarized \(V_A\) and \(V_B\) (\(\Delta V_A = 6.68 \pm 2.51\) mV; \(\Delta V_B = 7.22 \pm 2.68\) mV), and increased both TEP and RT (\(\Delta TEP = 0.59 \pm 0.27\) mV; \(\Delta R_T = 46 \pm 13\) \(\Omega\)·cm\(^2\)), consistent with inhibition of basolateral K-conductance. In five experiments,
basal \( \text{Ba}^{2+} \) (2 mM) reduced apical lactate-induced \( V_A \) and \( V_B \) depolarization in P1 by \( \approx 30\% \), whereas \( V_A \) and \( V_B \) hyperpolarization in P2 were unaffected (Table 4-5). Furthermore, basal \( \text{Ba}^{2+} \) also reduced lactate-induced TEP and \( R_T \) responses (Table 4-5), but it did not affect lactate-induced increase in \( R_A/R_B \). These observations suggest that apical lactate inhibited a \( \text{Ba}^{2+} \)-sensitive K-channel at the basolateral membrane. However, basal \( \text{Ba}^{2+} \) did not eliminate the lactate induced \( R_T \) response, suggesting that apical lactate increases \( R_T \) via other mechanisms. Possible lactate-activated mechanisms that can increase \( R_T \) are presented in the next section (section 4-5).

If apical lactate-induced activation of ClC-2 and apical membrane K-channel, and inhibition of basolateral membrane K-channel caused all the changes observed in \( V_A \), \( V_B \), TEP, and \( R_T \), the simultaneous inhibition of all these channels should completely eliminate these responses. This notion was tested by adding 2 mM \( \text{Ba}^{2+} \) (both apical and basal baths) and 200 \( \mu \text{M} \) \( \text{Zn}^{2+} \) (basal bath) simultaneously (Fig. 4-20). In the presence of \( \text{Ba}^{2+} \) and \( \text{Zn}^{2+} \), the lactate-induced TEP and \( R_A/R_B \) responses were completely eliminated, and the \( R_T \) response was increased by \( \approx 40\% \) (Table 4-5). \( V_A \) and \( V_B \) depolarization in P1 were both significantly reduced but not eliminated (Table 4-5). In addition, the \( V_A \) and \( V_B \) hyperpolarization in P2 were significantly reduced.
Fig. 4-20: Apical lactate induced $V_A$, $V_B$, TEP, $R_{RA/RB}$, $R_T$ responses in the presence of both $Zn^{2+}$ (basal bath) and $Ba^{2+}$ (apical and basal baths).

Although the addition of $Ba^{2+}$ and $Zn^{2+}$ completely eliminated the lactate induced TEP response, the $V_A$ and $V_B$ depolarization in P1 was not completely eliminated, albeit reduced. Electrical shunting between apical and basolateral membranes cannot account for this observation (all hfRPE have $R_T > 500 \ \Omega \cdot \text{cm}^2$). The equivalent increase in both $V_A$ and $V_B$ (without any $\Delta$TEP) is consistent with a change in liquid junction potential at the microelectrode tip, possibly due to a decrease in $[Cl]_i$. Apical lactate can decrease $[Cl]_i$ by increasing $Cl$-efflux via ClC-2, and causing cell-swelling, which dilutes the cytosol. However, blocking ClC-2 with $Zn^{2+}$ did not eliminate lactate-induced $V_A$ and
VB responses in P1, therefore cell-swelling probably generated the junction potential in P1.

Besides K-channels and ClC-2, other transport mechanism may be involved in lactate transport. A recent study showed that SMCT1, an electrogenic Na-linked lactate co-transporter (2:1 Na/Lac), is expressed exclusively at RPE basolateral membrane (Martin et al., 2007). However, our data suggest that SMCT1 contributes little to lactate transport: (1) thermodynamics predict that the large inward Na-gradient across the basolateral membrane prevents Na/lactate efflux via SMCT1; (2) 2Na/Lac efflux from the basolateral membrane via SMCT1 should decrease TEP, but apical lactate increased TEP; (3) the lactate-induced TEP response was completely blocked by Ba$^{2+}$ (apical and basal) and Zn$^{2+}$ (basal) – no other mechanisms contributed to this TEP response.

In summary, based on the following evidence, we show that apical lactate stimulated apical membrane K-channels, which hyperpolarize $V_A$ throughout P1 and P2 and increases TEP: (1) $V_A$ depolarization in P1 was larger in the presence of apical Ba$^{2+}$, whereas $V_B$ depolarization was unaffected; (2) the lactate-induced TEP response was smaller in the presence of apical Ba$^{2+}$; (3) the $V_A$ and $V_B$ hyperpolarization in P2 were reduced by apical Ba$^{2+}$. In addition, inhibition of either apical K-efflux (with Ba$^{2+}$) or basolateral Cl-efflux (via ClC-2; with Zn$^{2+}$) reduces the $V_A$ and $V_B$ hyperpolarization in P2 phase, suggesting that the activities of these two channels are electrically coupled to preserve charge neutrality. Surprisingly, apical lactate decreased $R_A/R_B$ in the presence of apical Ba$^{2+}$ (lactate increases $R_A/R_B$ in control) (Table 4-5). This is unusual because if
lactate activates apical K-channel (which decreases $R_A$ and reduces $R_A/R_B$), K-channel inhibition with apical $\text{Ba}^{2+}$ should amplify the lactate-induced increase in $R_A/R_B$. We postulate that the large $V_A$ and $V_B$ depolarization caused by apical $\text{Ba}^{2+}$ increases basolateral membrane K-channel conductance and activity (assuming outwardly rectifying channel). In its highly active state, its inhibition by lactate would cause a more pronounced increase in $R_B$, thus producing a significant decrease in $R_A/R_B$. In support of this hypothesis, this lactate-induced decrease in $R_A/R_B$ (in the presence of apical $\text{Ba}^{2+}$) was eliminated by basal $\text{Ba}^{2+}$.

The following results support the notion that lactate inhibits a K-channel at the basolateral membrane: (1) both $V_A$ and $V_B$ depolarization in P1 were reduced by basal $\text{Ba}^{2+}$; (2) lactate-induced TEP and $R_T$ responses were reduced by basal $\text{Ba}^{2+}$. However, evidence suggests that this K-channel is normally less active than apical membrane K-channel and ClC-2 because: (1) inhibition of basolateral K-channel contributes to a decrease in $R_A/R_B$ by increasing $R_B$, but lactate produced a net increase in $R_A/R_B$; (2) lactate-induced $R_A/R_B$ increase was unaffected by basal $\text{Ba}^{2+}$. The effect of basal $\text{Ba}^{2+}$ on apical membrane K-channel and ClC-2 is minimal because basal $\text{Ba}^{2+}$ did not affect $V_A$ and $V_B$ hyperpolarization in P2. Next, we show that the inhibition of all these channels (i.e., apical and basolateral membrane K-channels, and ClC-2) eliminates the lactate induced TEP response, but did not affect lactate-induced $V_A$ and $V_B$ depolarization. This observation suggests that lactate caused cell swelling, which is discussed further in the next section.
**Section 4.4 conclusions:** Apical lactate activates Kir 7.1 K-channel at the apical membrane.  
Kir 7.1 and CIC-2 activity is electrically coupled.  
Apical lactate inhibits a Ba^{2+}-sensitive K-channel at the basolateral membrane.

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Section 4.6 – Mechanism of lactate induced ion-channel activation or inhibition

The lactate-induced changes in K-channel and ClC-2 activities may be caused by cell swelling or acidification. As previously discussed, microelectrode experiments suggest that apical lactate caused cell-swelling, which may activate these ion-channels.

Mammalian cells respond to hypoosmotic challenge by activating K- and Cl- channels to drive KCl and fluid out of the cell (Nilius et al., 1997; Eggermont et al., 2001). The RPE expresses BK-channel (Ca$^{2+}$-activated K-channel) and ClC-2 (Grunder et al., 1992; Furukawa et al., 1998; Xiong et al., 1999; Sheu et al., 2004) – both are swell-activated ion-channels and may participate in regulatory volume decrease.

![Fig. 4-21: Apical lactate vs. hyperosmotic Ringer solution induced cell-swelling.](image)

To better quantify lactate-induced cell swelling, we used a pH$_i$-insensitive dye (calcein) with a three-point osmolarity calibration (with 280, 330, 355 mOsm Ringer) to show that
apical lactate caused cell-swelling equivalent to $8 \pm 2$ mOsm ($n = 6$; Fig. 4-21). If apical membrane K-channels and ClC-2 are indeed activated by cell-swelling, decreasing Ringer osmolarity (from 305 to 280 mOsm; both solution baths) should increase TEP. However, hypoosmotic Ringer decreased TEP by $0.28 \pm 0.16$ mV, whereas lactate increased TEP by $1.06 \pm 0.34$ mV ($n = 6$). Furthermore, hypo-osmotic Ringer did not affect $R_T$. These observations suggest that cell-swelling did not activate apical K-channel or basolateral K-channel and ClC-2.

Fig. 4-22: Lactate, low pH (6.8), and hypotonic induced pHi, TEP, and $R_T$ responses.

Alternatively, apical lactate may stimulate these K-channels and ClC-2 by causing cell acidification. Kir 7.1 K-channels are highly expressed in RPE apical membrane and is activated by mild intracellular acidification (Yuan et al., 2003; Hughes & Swaminathan,
In addition, CLC-2 is stimulated by acidic conditions (Jordt & Jentsch, 1997; Hartzell & Qu, 2003). Consistent with this notion, we show that acidifying the cell by reducing bath pH (pH 6.8; both solution baths) produced a TEP response (0.83 ± 0.45 mV) with a magnitude comparable to that caused by 20 mM apical lactate (ΔTEP = 1.20 ± 0.52 mV; n = 5; p < 0.05) (Fig. 4-22). However unlike lactate, low pH Ringer had no effect on R_T. If cell acidification stimulated CLC-2 activity, we should be able to block low pH Ringer-induced TEP response with basal Zn^{2+} (200 μM) (Fig. 4-23).

![Figure 4-23: pH 6.8 Ringer induced pH_i, TEP, and R_T responses in the presence of basal Zn^{2+}.](image)

Consistent with our hypothesis, Zn^{2+} reduced the low pH-induced TEP response by more than 2-fold (from ΔTEP = 1.04 ± 0.47 to 0.35 ± 0.13 mV; n = 5; p < 0.05) – this effect was irreversible. Although low pH Ringer induced acidification was unaffected by basal
Zn\(^{2+}\), upon returning to control Ringer (switched from low pH Ringer), cell pH\(_{i}\) was unable to return to baseline – consistent with the irreversible inhibitory effect of Zn\(^{2+}\) on NHE. Since apical membrane K-channels are electrically coupled to ClC-2 activity, we tested if lactate-induced cell acidification activated apical membrane K-channels. Low pH Ringer (both solution baths) induced pH\(_{i}\), TEP, and R\(_T\) responses were measured in the presence or absence of 2 mM apical Ba\(^{2+}\) (Fig. 4-24).

Fig. 4-24: pH 6.8 Ringer induced pH\(_{i}\), TEP, and R\(_T\) responses in the presence of apical Ba\(^{2+}\).

Interestingly in the presence of apical Ba\(^{2+}\), low pH Ringer decreased TEP (ΔTEP = -0.31 ± 0.12 mV), whereas in absence of Ba\(^{2+}\), low pH Ringer increased TEP (ΔTEP = 0.80 ± 0.42 mV; n = 5; p < 0.05). The low pH Ringer induced pH\(_{i}\) response was unaffected by
apical Ba\textsuperscript{2+}, however upon returning to control Ringer, the recovery to baseline pH\textsubscript{i} was slower compared to control.

Finally, we test the pH\textsubscript{i}-sensitivity of the basolateral membrane K-channel; low pH Ringer was perfused to both apical and basal baths in the presence or absence of 2 mM basal Ba\textsuperscript{2+} (Fig. 4-25). Unexpectedly, the low pH Ringer-induced TEP response slightly increased in the presence of basal Ba\textsuperscript{2+} (from $\Delta$TEP = 1.14 ± 0.37 to 1.45 ± 0.40 mV; n = 5; p < 0.05). This experiment indicates that cell acidification did not inhibit basolateral membrane K-channels.
Collectively, our data indicates that cell acidification activates apical membrane K-channels and basolateral membrane ClC-2, but does not inhibit basolateral membrane K-channel (Fig. 4-26). This is also consistent with the notion that lactate-induced inhibition of basolateral membrane K-channel is not as significant as its activation of apical membrane K-channel and ClC-2. The observations that either basal Zn^{2+} or apical Ba^{2+} can almost completely block the low pH Ringer-induced TEP response corroborate our conclusion in section 4.4 that KCl-efflux via these two channels are electrically coupled. The apical lactate-stimulated apical membrane K-channel is most likely Kir 7.1 because (1) it is activated by intracellular acidification with maximal activity at pH 7.1 (Yuan et al., 2003) and (2) it is the major K-conductance at the apical membrane of the RPE (Shimura et al., 2001; Yang et al., 2003; Yang et al., 2008b).

Fig. 4-26: Lactate-induced ion transport mechanisms in RPE. Lactate entry via MCT1 activates NHE and Kir 7.1 at the apical membrane, and ClC-2 at the basolateral membrane. Lactate inhibits a basolateral membrane K-channel via an unknown mechanism.
Although apical lactate inhibited a basolateral membrane K-channel, this channel is not pH$_i$-sensitive because basal Ba$^{2+}$ had no effect on low pH Ringer-induced TEP response. Furthermore, low pH Ringer did not appreciably increase R$_T$. We also show that this K-channel is not Ca$^{2+}$-dependent: BAPTA-AM (intracellular Ca$^{2+}$ chelator) had no effect on lactate-induced TEP and R$_T$ responses. Collectively, our data indicates that lactate activates a Ba$^{2+}$-sensitive, pH-insensitive, and outwardly rectifying K-channel at the basolateral membrane, whose activity has been demonstrated in RPE cells (Strauss et al., 1993; Hughes et al., 1995; Strauss et al., 2002).

Apical lactate caused a large increase in R$_T$ and is consistent with inhibition of basolateral membrane K-channel, but this R$_T$ response was only modestly reduced by basal Ba$^{2+}$. In addition, this R$_T$ response is time-delayed relative to V$_A$, V$_B$, and TEP responses – R$_T$ began to increase at the start of P2. However, lactate-induced inhibition of basolateral membrane K-channel occurs in P1, suggesting that other mechanisms caused this R$_T$ response. Although apical lactate caused cell swelling and intracellular acidification, neither hypo-osmotic challenge nor extracellular acidification affected R$_T$. One possibility is that lactate increases R$_T$ by increasing tight junction resistance, perhaps by interacting with tight junction proteins or its adaptors. Alternatively, carboxylates may be the main effector of the R$_T$ response since other monocarboxylates (e.g., pyruvate, acetate, and propionate) also increased R$_T$. 
Section 4.5 conclusions: Apical lactate causes cell swelling
Intracellular acidification activates Kir 7.1 and CIC-2

Supporting experimental observations:
- Zn$^{2+}$ and Ba$^{2+}$ did not eliminate lactate-induced $V_A$ and $V_B$ depolarization (in P1)
- Hypo-osmotic solution and lactate alters calcine fluorescence signals in the same direction
- Hypo-osmotic solution causes cell-swelling but decreased TEP (lactate increased TEP)
- Low pH (6.8) Ringer increases TEP
- Basal Zn$^{2+}$ blocks low pH (6.8) Ringer-induced TEP response
- Apical Ba$^{2+}$ blocks low pH (6.8) Ringer-induced TEP response
- Basal Ba$^{2+}$ did not affect low pH (6.8) Ringer-induced TEP response

Interpretation:
- Lactate causes microelectrode junction potential, indicating cell-swelling
- Lactate causes cell swelling
- Cell swelling did not activate Kir 7.1 or CIC-2
- Cell acidification activates electrogenic mechanisms
- Cell acidification activates CIC-2
- Cell acidification activates Kir 7.1
- Cell acidification did not inhibit basolateral membrane K-channel
Section 4.7 – Lactate, HCO$_3^-$, and fluid transport in RPE

In addition to lactate-induced activation of KCl transport, the CO$_2$/HCO$_3^-$ buffering system in the RPE helps regulate cell volume and pH$_i$ (Kenyon et al., 1997). Early studies show that MCT1 interacts with HCO$_3^-$-transporters (Becker et al., 2004) and carbonic anhydrases (CAs) (Wetzel et al., 2001; Becker et al., 2005). Preliminary data shows that the apical membrane Na/2HCO$_3^-$ co-transporter (NBC1) and HCO$_3^-$-buffering by CAs facilitates lactate-transport across RPE (Adijanto et al, ARVO 2009). The interactions between MCT1, NBCs, and CAs in RPE are currently being studied.

In RPE, the activity of the apical membrane Na/K ATPase increases in the dark (Griff et al., 1985) due to a higher SRS [K$^+$] level ($\approx$ 5 mM) in the dark than in light ($\approx$ 2 mM). A high SRS [K$^+$] in the dark is due to: (1) active K-channel activity at photoreceptor inner segments (Gallemore et al., 1998); (2) CO$_2$-driven fluid transport across the RPE dehydrates the SRS, thus increasing SRS [K$^+$] (see Chapter 3); (3) lactate-induced increase in Kir 7.1 conductance at the apical membrane increases SRS [K$^+$]. By increasing SRS [K$^+$] and Na/K ATPase activity, lactate enhances CO$_2$/HCO$_3^-$ transport in two ways: (1) Na/K ATPase activity maintains a favorable Na$^+$-gradient that facilitates NBC1-mediated HCO$_3^-$-transport across the apical membrane (Adijanto et al., 2009); (2) increased Kir 7.1 and Na/K ATPase activity increases TEP, which facilitates paracellular Na-absorption – Na acts as the counter-ion for transcellular HCO$_3^-$-transport.

Since lactate is transported across the RPE in addition to facilitating HCO$_3^-$-transport, the addition of lactate to the apical bath should increase steady-state fluid transport across the
tissue. Assuming that all lactate produced by photoreceptors (0.137 μmol/min; (Wang et al., 1997b)) is transported across the RPE, we calculated that lactate-absorption should be accompanied by ≈ 2.5 μL/cm²·hr of fluid across the tissue to maintain osmotic balance. However, adding lactate to the apical bath did not affect steady-state fluid absorption in any RPE preparations tested (e.g., bovine, frog, human, and cultured hfRPE) (data not shown), suggesting that apical lactate may counteract the osmotic effects of its absorption by activating solute-secreting mechanisms.

The RPE maintains pH homeostasis in the SRS by transporting photoreceptor generated CO₂ and lactate to the choroidal blood supply. In the dark adapted eye, photoreceptors produce large amounts of CO₂ and lactate, which are released into the SRS – accumulation of these metabolic by-products in RPE causes cell swelling and osmotic stress. In this study, we demonstrate how the RPE decreases cell volume to mitigate this osmotic stress, by using lactate to activate KCl efflux via Kir 7.1 and ClC-2 at the apical and basolateral membranes respectively. In addition, lactate-induced ClC-2 activation may help regulate ion-transport at the paracellular pathway. In support of this notion, mice lacking ClC-2 expression exhibit reduced TEP and has a smaller short-circuit current across the RPE (Bosl et al., 2001). These mice also develop early onset retinal degeneration. Therefore, lactate-induced activation of Kir 7.1 and ClC-2 may help prevent osmotic stress-induced RPE damage, thus maintaining photoreceptor health and integrity.
CHAPTER 5: Lactate and CO₂/HCO₃ Transport in RPE

Section 5.1 – Introduction

The concept of acid-base coupled H₂CO₃-transport via MCT1 and NBC1 has been demonstrated in Xenopus oocytes (Becker et al., 2004; Becker & Deitmer, 2004). The localization and activity of MCT1, NBC1, and NBC3 at the RPE apical membrane (Hughes et al., 1989; Bok et al., 2001; Philp et al., 2003b; Adijanto et al., 2009) suggests that these transporters may be acid-base coupled, and are therefore functionally dependent on each other. In this model, HCO₃ transport via NBC1 buffers protons entering the cell via MCT1, thus maintaining a favorable H⁺-gradient across the cell that increases lactate transport (see Fig. 1-6). Furthermore, carbonic anhydrases (CAs) catalyze the hydration/dehydration reaction between CO₂ and HCO₃⁻, which has been shown to enhance the activity of MCT1 (Wetzel et al., 2001; Becker et al., 2005; Svichar et al., 2006) and NBCs (Alvarez et al., 2003; Loiselle et al., 2004; McMurtrie et al., 2004; Pushkin et al., 2004). Since membrane bound CA (CA XIV) is localized at the RPE apical membrane (Nagelhus et al., 2005), it may functionally interact with MCT1 and NBC1 to enhance both lactate and HCO₃ transport into the cell at the apical membrane.

In chapter 4, we show that lactate stimulates several ion transport mechanisms in RPE (Fig. 4-26). Conducting these experiments in CO₂/HCO₃-free (HEPES buffered) conditions simplifies the lactate transport system, but this maneuver eliminates potential MCT1 interactions with HCO₃-dependent mechanisms such as the electroneutral Na/HCO₃ co-
transporter (NBC3), and carbonic anhydrases (Wetzel et al., 2001; Becker et al., 2005).

In addition to K and Cl channel mediated volume regulation (see Chapter 4), the CO$_2$/HCO$_3$ buffering system in the RPE helps regulate cell volume and pH$_i$ (Kenyon et al., 1997).

In this chapter, we investigate interactions between MCT1 and NBCs, and test the role of CAs in a “lactate-bicarbonate-transport metabolon”. We show that MCT1-mediated H/Lac co-transport enhances HCO$_3$-transport via NBC1 and NBC3 at the apical membrane. We also show that H$^+$-buffering by these HCO$_3$-transporters and carbonic anhydrases facilitates lactate-transport across RPE. Interactions between NBC1, NBC3, MCT1, and CAs are synergistic, thus maximizing lactate, HCO$_3$, and fluid transport across the apical membrane. In the next section, we demonstrate that lactate is transported across the basolateral membrane mainly by AE2, with some contribution from MCT3 & MCT4.
Section 5.2 – Lactate and HCO$_3$ transport at the RPE apical membrane

As demonstrated in chapter 4, lactate transport across the apical membrane is mediated by MCT1. To study the interactions between lactate and HCO$_3$ transport mechanisms in RPE, we compared the effect of iso-osmotic addition of 20 mM lactate (pH 7.4) into the apical bath in the presence vs. absence of CO$_2$/HCO$_3$ (Fig. 5-1).

![Fig. 5-1: Apical lactate induced pH$_i$, TEP, and $R_T$ responses in the presence vs. absence of CO$_2$/HCO$_3$. Red box – R1 phase; blue box – R2 phase.](image)

In either case, apical lactate produced a pH$_i$ response with two phases (Fig. 5-1): a fast acidification (R1) followed by a slow alkalinization (R2). R1 reflects H$^+$-coupled lactate entry via MCT1 (Figs. 4-3 and 4-4) and R2 reflects the activity of H$^+$-buffering mechanisms such as the Na/H exchanger (Fig. 4-5). R1 in the presence of CO$_2$/HCO$_3$ was $\approx$ 2-fold larger than in the absence of CO$_2$/HCO$_3$ (-12.80 ± 4.33 vs. -6.32 ± 1.46
mM·min⁻¹; p < 0.01; n = 59), whereas R₂ was ≈ 20% larger in the presence vs. absence of CO₂/HCO₃ (0.86 ± 0.35 vs. 0.71 ± 0.31 mM·min⁻¹; p < 0.01; n = 59) – both observations are consistent with the notion that HCO₃⁻-transport mechanisms are activated to help buffer H⁺-coupled lactate-entry via MCT1. Furthermore, lactate-induced TEP and Rₜ responses were respectively ≈ 30% and 40% larger in the absence of CO₂/HCO₃ (∆TEP = 1.39 ± 0.60 vs. 0.98 ± 0.41 mV; ∆Rₜ = 52 ± 21 vs. 31 ± 14 Ω·cm²; p < 0.01; n = 59), suggesting that lactate activated HCO₃⁻-independent electrogenic mechanisms. This is consistent with our earlier finding: in the absence of CO₂/HCO₃, lactate activates apical membrane Kir 7.1 K-channels and basolateral membrane ClC-2 Cl-channel (sections 4.4 & 4.5) by causing cell acidification (section 4.6; Fig. 4-26).

Fig. 5-2: (In CO₂/HCO₃ Ringer) Apical lactate-induced pHi, TEP, and Rₜ responses in the presence of Zn²⁺ (basal) and Ba²⁺ (apical).
To test if lactate activates Kir 7.1 and CLC-2 in the presence of CO₂/HCO₃, we measured lactate-induced TEP response in the presence of Zn²⁺ (basal) and Ba²⁺ (apical) (Fig. 5-2). Surprisingly, Zn²⁺ and Ba²⁺ did not reduce lactate-induced TEP response in the presence of CO₂/HCO₃ (∆TEP = 0.39 ± 0.13 vs. 0.54 ± 0.19; p > 0.05; n = 3). This observation suggests that lactate activated a HCO₃-dependent electrogenic mechanism that increases TEP. A possible candidate is the DIDS-sensitive and electrogenic Na/2HCO₃ co-transporter (NBC1) at the RPE apical membrane (Figs. 1-8 & 5-5).

![Graph showing pH and TEP responses](image)

Fig. 5-3: (In CO₂/HCO₃ Ringer) Apical lactate-induced pHᵢ, TEP, and Rᵢ responses in the presence of apical DIDS.

To test if lactate activates NBC1, we added 0.5 mM DIDS into the apical bath (Fig. 5-3) and found that the lactate-induced TEP response was significantly reduced from 0.82 ±
0.44 to 0.36 ± 0.33 mV (n = 12) and that the R_T response was increased by almost 2-fold (from 27 ± 14 to 50 ± 20 Ω·cm²; n = 12; p < 0.05). These observations suggest that NBC1-mediated HCO₃⁻ transport helps buffer H⁺-entry into the cell via MCT1.

Interestingly, R2 of the pH_i response was 36% larger in the presence of DIDS compared to control (R2 = 0.92 ± 0.52 vs. 0.68 ± 0.37 mM·min⁻¹; p < 0.05; n = 12), suggesting that other H⁺-buffering mechanisms (e.g., NHE) helped compensate for the loss of NBC1 activity.

Fig. 5-4: Lactate induced ion-transport mechanisms in RPE (in the presence of CO₂/HCO₃): Lactate activates NBC1 and NBC3 (blue box), and Kir 7.1 and ClC-2 (orange box).

These experiments suggest that apical lactate can increase TEP by activating two separate mechanisms (Fig. 5-4): (1) lactate-transport via MCT1 drives apical Na/2HCO₃ co-transport into the cell via NBC1; (2) lactate-entry into the cell activates apical membrane
K-channel (Kir7.1) and basolateral membrane Cl-channel (ClC-2), and inhibits basolateral membrane K-channel (Figs. 4-20 & 4-26).

Fig. 5-5: (In CO₂/HCO₃ Ringer) Apical lactate induced pHᵢ, TEP, and Rᵣ responses in the presence of Zn²⁺ (basal) and Ba²⁺ (apical & basal), then apical DIDS.

However, as shown in Fig 5-2 and 5-5, apical lactate-induced TEP response (ΔTEP = 1.36 ± 0.41 mV) was not blocked by the simultaneous addition of Ba²⁺ (apical & basal) and Zn²⁺ (basal) (ΔTEP = 1.68 ± 0.74 mV; p > 0.05); in some cases, the TEP response was larger in the presence of Ba²⁺ and Zn²⁺ (3 of 6 tissues). This observation does not necessarily imply that lactate does not activate K-channels and ClC-2 (in the presence of
CO$_2$/HCO$_3$) because the Ba$^{2+}$-induced $V_A$ depolarization may create an electrical gradient that promotes NBC1 activity. We verified this hypothesis by showing that subsequent addition of DIDS to the apical bath (still in the presence of Ba$^{2+}$ and Zn$^{2+}$) completely eliminated the lactate-induced TEP response ($p < 0.05$; Fig 5-5).

Since NBC1 is Na-linked, Na-removal from both bathing solutions should block lactate-induced TEP response (Fig. 5-6). In seven experiments, apical lactate-induced TEP response was essentially abolished upon Na-removal from both apical and basal baths. Furthermore, R2 was completely eliminated, which corroborates the notion that Na-coupled HCO$_3$-transporter mediates H$^+$-buffering in RPE. However, NBC1 is not the
only Na-linked pH$_i$-regulatory mechanism in RPE: other mechanisms include NBC3 (Na/HCO$_3$ co-transporter) and NHE (Na/H exchanger) (see Fig. 1-8).

![Graph showing pH$_i$, TEP, and RT responses in presence of apical amiloride. Red box – R1 phase; blue box – R2 phase.}

In chapter 4 (section 4.3), we showed that apical lactate activates the Na/H exchanger (NHE) at RPE apical membrane. NHE activity is reflected by R2 of the lactate-induced pH$_i$ response, which is blocked by amiloride (Fig. 4-5). However in the presence of CO$_2$/HCO$_3$, amiloride reduced R2 by only 50% (R2 from 0.77 ± 0.13 to 0.38 ± 0.22 mM·min$^{-1}$; p = 0.046; n = 5; Fig. 5-7) – it did not eliminate R2. In addition, amiloride had no effect on R1 (p > 0.05), suggesting that additional pH-regulatory mechanisms (i.e., HCO$_3$-transporters, possibly NBC1) are actively participating in H$^+$-buffering. To
test this notion, we simultaneously inhibit NHE and NBC1 with Zn$^{2+}$ and DIDS, respectively (Fig. 5-8). Amiloride and Zn$^{2+}$ are both NHE inhibitors, and Zn$^{2+}$ was used in this experiment because amiloride reacts covalently with DIDS to form a light-yellow precipitate (data not shown).

Like amiloride, apical Zn$^{2+}$ per se had little effect on R2 ($p > 0.05$; $n = 5$). In addition, the presence of both Zn$^{2+}$ and DIDS in the apical bath to block both NBC1 and NHE had little effect on R2 ($p > 0.05$; $n = 3$; Fig. 5-7), suggesting that the DIDS-insensitive Na/HCO$_3$ co-transporter (NBC3) may be actively transporting HCO$_3$ into the cell. To test this notion, we inhibit HCO$_3$-transporters (NBC1 and NBC3) by using
ethoxyzolamide (EZA; 10 μM), a membrane-permeable CA-inhibitor, to non-selectively block all cytosolic and membrane bound CAs (Fig. 5-9).

Fig. 5-9: (In CO₂/HCO₃ Ringer) Apical lactate induced pHᵢ, TEP, and Rₜ responses in presence of ethoxyzolamide (EZA) followed by amiloride in the apical bath. Red box – R1 phase; blue box – R2 phase.

In the presence of EZA in the apical bath, lactate caused larger pHᵢ (R1 & R2), TEP, and Rₜ responses compared to control (p < 0.05; n = 4). Although EZA increased R2 (from 1.03 ± 0.35 to 1.59 ± 0.58 mM·min⁻¹), subsequent addition of amiloride further reduced and eliminated R2 (from 1.59 ± 0.58 to 0.13 ± 0.51 mM·min⁻¹; p < 0.05; n = 4) – indicating that apical lactate activates NBC1, NBC3, and NHE at the apical membrane.
Although lactate-induced TEP response is larger in the presence of EZA ($\Delta$TEP = 3.39 ± 1.22 vs. 1.60 ± 0.55 mV; p < 0.05; n = 4), this observation does not indicate increased NBC1 activity – the elimination of R2 by EZA and amiloride demonstrates that EZA inhibits NBC1. To verify this hypothesis, we compared lactate-induced TEP response in the presence of apical DIDS vs. in the presence of both DIDS and EZA (Fig 5-10).

In six experiments, EZA increased lactate-induced TEP response even in the presence of apical DIDS ($\Delta$TEP from 0.96 ± 0.86 to 1.62 ± 0.62 mV; p < 0.05), suggesting that EZA enhanced lactate-induced activation of HCO₃⁻-independent mechanisms. Since lactate stimulates Kir7.1 and ClC-2 via cell acidification, EZA may enhance lactate-induced
activation of these channels by amplifying lactate-induced cell acidification (EZA decreases intracellular buffering capacity). Consistent with this notion, in the presence of apical EZA, apical lactate-induced TEP response was reduced by Ba$^{2+}$ (apical & basal baths) and Zn$^{2+}$ (basal bath) (from 2.32 ± 0.58 to 1.60 ± 0.56 mV; n = 4; p < 0.05) (Fig 5-11). The R2 phase of the pH response was completely eliminated in the presence of EZA and Zn$^{2+}$ (p < 0.05). This observation corroborates our earlier conclusion that (in the presence of CO$_2$/HCO$_3$) apical lactate stimulates NBC1, NBC3, and NHE at the apical membrane.

Fig. 5-11: (In CO$_2$/HCO$_3$ Ringer) Apical lactate induced pH$_i$, TEP, and R$_T$ responses in presence of apical ethoxyzolamide (EZA) followed by Zn$^{2+}$ (basal) and Ba$^{2+}$ (apical & basal). Red box – R1 phase; blue box – R2 phase.
Collectively, our data show that indiscriminate inhibition of all CAs in the RPE reduces NBC1 and NBC3 activity. However, how much does membrane-bound CAs contribute to NBC-mediated HCO₃⁻-transport compared to cytosolic CAs? To answer this question, we added membrane-impermeable CA-inhibitor, benzolamide (BZA; 10 μM), to the apical bath (Fig. 5-12).

![Fig. 5-12: (In CO₂/HCO₃ Ringer) Apical lactate induced pHi, TEP, and Rₜ responses in the presence of apical benzolamide (BZA).](image)

In seven experiments, apical lactate induced R1 and TEP responses were ≈ 40% and ≈ 30% larger compared to control, respectively (p < 0.05). However, unlike EZA, BZA had little effect on lactate-induced pHᵢ or Rₜ responses. Further, the simultaneous addition of BZA and amiloride to the apical bath did not reduce R2 of the lactate-induced**
pH response. These experiments suggest that cytosolic CAs play a more prominent role in facilitating HCO₃⁻-transporter activity than membrane-bound CAs.

Based on the experiments presented in this section, we showed that MCT1 is acid-base coupled with NBC1: (1) in the absence of CO₂/HCO₃, both R1 and R2 were smaller than in the presence of CO₂/HCO₃, suggesting that HCO₃⁻-transport helps buffer H⁺-coupled lactate-entry via MCT1; (2) Na-removal from both solution baths eliminates NBC1, NBC3, and NHE activities, thus blocking both R2 and TEP response; (3) apical lactate-induced TEP response was significantly reduced by apical DIDS; (4) this TEP response was completely blocked by the simultaneous addition of Zn²⁺ (basal), Ba²⁺ (apical & basal), and apical DIDS; (5) EZA or amiloride per se modestly reduced R2, but R2 is completely eliminated by the simultaneous addition of EZA and amiloride. At steady-state, lactate that enters the cell from the apical membrane must exit the basolateral membrane; this process is discussed in the following section.
**Section 5.1 conclusions:** Apical H/Lac entry via MCT1 activates NHE, NBC1, and NBC3 to regulate pH, H/Lac transport via MCT1 drives NBC1 and NBC3 activity with the help of CA differences.

**Supporting experimental observations**

- R2 was present in the presence or absence of CO₂/HCO₃⁻
- R2 was larger in the presence vs. in the absence of CO₂/HCO₃⁻
- Na-removal from both solution baths eliminates R2
- Blocking NHE with amiloride decreased R2
- Blocking NHE with apical Zn²⁺ per did not affect R2
- Blocking NBC1 with apical DIDS did not decrease R2
- Adding both apical Zn²⁺ and DIDS did not affect R2
- Inhibiting CAs with EZA (inhibits HCO₃⁻-transporters) decreased R2
- Apical lactate-induced TEP response was reduced by apical DIDS

**Interpretation**

- Lactate activates NHE
- Lactate activates NBC1 and/or NBC3
- Lactate activates NBC1 and/or NBC3
- Lactate activates NBC3 and/or NHE
- Lactate activates NBC1 and NBC3
- Lactate activates NBC1, NBC3, and NHE
- TEP response is mediated by a Na-dependent mechanism
- Part of this TEP response reflects NBC1 activity

**Fig.**

- 5-1
- 5-7
- 5-8
- 5-9
- 5-6
- 5-9
- 5-3
Section 5.3 – Lactate and HCO$_3^-$ transport at the RPE basolateral membrane

As discussed in section 1.5, lactate transport across the RPE is mediated by MCT1 at the apical membrane, and MCT3, MCT4, and AE2 at the basolateral membrane (see Fig. 1-10). At the basolateral membrane, AE2 can facilitate lactate transport in two ways (Fig. 5-13): First, HCO$_3^-$-transport via AE2 can participate in acid-base coupling with MCT3 and MCT4 (H/Lac transport) (Fig. 5-13; yellow box). Alternatively, AE2 can mediate Cl/Lac exchange to directly transport lactate out of the cell (Fig. 5-13; blue box).

To study AE2-mediated lactate and HCO$_3^-$ transport, we added lactate to the basal bath – lactate entry into the cell via MCT3 or MCT4 should cause cell acidification, whereas lactate transport via AE2 should not affect pH$_i$. However, as shown in Fig. 5-14, adding...
lactate to the basal bath caused cell \textit{alkalinization} and decreased steady-state TEP and $R_T$ ($\Delta \text{pH}_i = 0.04 \pm 0.01$, $n = 11$; $\Delta \text{TEP} = -0.38 \pm 0.18$ mV, $n = 13$). The $R_T$ response however, was small and insignificant. Since AE2 can be blocked by DIDS (Fig. 3-13), we added DIDS to the basal bath and showed that DIDS completely eliminated basal lactate-induced alkalinization ($p < 0.05$; $n = 6$).

AE2-mediated lactate-transport does not directly cause cell alkalinization, thus suggesting involvement of other pH-dependent mechanisms. In this regard, we eliminated HCO$_3$-transport mechanisms by showing that in the absence of CO$_2$/HCO$_3$ (HEPES buffered) adding lactate into the basal bath caused cell alkalinization, and this

![Graph showing pH, TEP, and R_T responses](image-url)
alkalinization was significantly reduced by basal DIDS (from $\Delta \text{pH}_i = 0.13 \pm 0.01$ to $0.05 \pm 0.01$; $n = 4$; $p < 0.01$; Fig. 5-15).

Fig. 5-15: (CO$_2$/HCO$_3$-free Ringer) Basal lactate-induced pH$_i$, TEP, and R$_T$ responses in the presence of basal DIDS.

We showed that basal lactate-induced alkalinization is DIDS-sensitive and HCO$_3$-independent, but lactate entry into the cell via AE2 should not alter pH$_i$, thus suggesting the involvement of a secondary mechanism. It is possible that lactate entering the cell via AE2 is transported out of the cell at the apical membrane via MCT1 (Fig. 5-18), thus causing cell alkalinization. We confirmed this possibility by showing that basal lactate-induced alkalinization (in control; $\Delta \text{pH}_i = 0.03 \pm 0.01$) was converted into cell
Acidification ($\Delta pHi = -0.03 \pm 0.01$) in the presence of 100 $\mu$M niflumic acid (MCT1-inhibitor; see Fig. 4-4) (Fig. 5-16).

Fig. 5-16: (In CO$_2$/HCO$_3$ Ringer) Basal lactate-induced pH$_i$, TEP, and R$_T$ responses in the presence of apical niflumic acid.

The basal lactate-induced pH$_i$ response (in the presence of niflumic acid) exhibits R1 and R2 phases (see section 4.3 and Fig. 4-2) similar to that caused by apical lactate. Hence, R1 is probably caused by H/Lac entry into the cell via MCT3 and MCT4, whereas R2 reflects activation of pH$_i$-regulatory mechanisms at the apical membrane (i.e., NHE, NBC1, and NBC3) and/or at the basolateral membrane (i.e., NBC). In the absence of CO$_2$/HCO$_3$ (HEPES-buffered), niflumic acid also converted the basal lactate-induced alkalinization into a pH$_i$ response with distinct R1 and R2 phases ($n = 5$; Fig. 5-17).
this case, R1 reflects MCT3 and MCT4 activity, and R2 can only be attributed to NHE activity.

Although MCT3, MCT4, and AE2 are all electroneutral processes, the data summarized in Figs 5-14 to -17 shows that adding lactate to the basal bath decreased TEP. This TEP response originates from basolateral membrane hyperpolarization (Kenyon et al., 1994), and this electrical response may derive from two possible mechanisms (see Fig. 5-18): (1) basal lactate entry via AE2 decreases [Cl], which reduces Cl-channel activity, and (2) basal lactate entry via MCT3 drives electrogenic Na/nHCO₃ co-transport into the cell via basolateral membrane NBC (acid-base coupling). Our data corroborated the former
mechanism since the inhibition of basal lactate-induced TEP response by basal DIDS (CO$_2$/HCO$_3$-free; Fig. 5-15) or niflumic acid (which inhibits Ca$^{2+}$-dependent Cl-channels (Hartzell et al., 2005); Fig. 5-16) is both consistent with involvement of Cl-channels.

Interestingly, Fig. 5-14 shows that basal lactate increased TEP (by 0.21 ± 0.12 mV) in the presence of basal DIDS (in CO$_2$/HCO$_3$ Ringer) – this did not occur in the absence of CO$_2$/HCO$_3$ (HEPES buffered; Fig. 5-15), thus suggesting the involvement of a HCO$_3$-transport mechanism. In this context, activation of NBC1 would hyperpolarize $V_A$ and

Fig. 5-18: Basal lactate-induced ion transport in RPE. At the basolateral membrane, MCT3, MCT4, and AE2 transports lactate into the cell. Lactate is subsequently transported out of the cell via MCT1 at the apical membrane. Lactate entry into the cell via AE2 reduces [Cl$^-$], which reduces Cl-channel activity. The basolateral membrane NBC may be acid-base coupled to MCT3 activity, and this interaction may be facilitated by carbonic anhydrase II. At the apical membrane, NBC1 and NHE activity buffers protons entering the cell via MCT3.
increase TEP (see Fig. 5-18). This observation suggests that basal lactate activates NBC1, but its electrical activity is normally masked by concomitant changes in Cl-channel activity (hyperpolarize V_B; decrease TEP). There may be functional interaction between MCT3 (and MCT4) and basolateral membrane NBC, but evidence to support this notion is lacking – this is caused by experimental difficulties due to the lack of specific NBC inhibitors (DIDS will block NBC, AE2, ClC-2 channel, and Ca^{2+}-activated Cl-channel at the basolateral membrane).

In summary, we demonstrate that functional interaction between NBC1, MCT1, and CAs enhances lactate, HCO_3, and fluid transport at the RPE apical membrane. At the basolateral membrane, AE2 mediates the major component of lactate transport (compared to MCT3 & MCT4). AE2-mediated lactate-efflux from the basolateral membrane drives Cl into the cell, which is subsequently recycled back out of the cell mainly via Ca^{2+}-activated Cl-channels (perhaps with minor contributions from CFTR) – this mechanism helps drive fluid across the basolateral membrane. We show how the RPE protects itself from acidosis and osmotic stress imposed by photoreceptor generated metabolic acids in light or in the dark. We also demonstrate how the RPE uses lactate-transport mechanisms and its interactions with pH_i and volume regulating mechanisms to maintain ion, volume, and pH homeostasis in the SRS, thus preserving the health and integrity of the neural retina.
Section 5.2 Conclusion: Cl/Lac exchange via AE2 is the major lactate transport mechanism at the basolateral membrane

Supporting experimental observations
Adding lactate into the basal bath caused alkalization
Basal lactate caused acidification in the presence of apical niflumic acid
Basal lactate caused acidification in the presence of apical niflumic acid
Basal DIDS blocked the basal lactate-induced alkalization
Adding lactate into the basal bath decreased TEP
Basal DIDS blocked the basal lactate-induced TEP decrease
Apical niflumic acid blocked the basal lactate-induced TEP decrease

Interpretation
Lactate entry from the basolateral membrane exits apical membrane via MCT1
Lactate entry from the basolateral membrane exits apical membrane via MCT1
MCT3 and MCT4 mediates lactate transport at the basolateral membrane
AE2 mediates lactate transport at the basolateral membrane
Basal lactate activated an electrogenic mechanism
Lactate transport via AE2 drives Cl-recycling by a DIDS-sensitive Cl-channel
Lactate transport via AE2 drives Cl-recycling by Ca<sup>2+</sup>-dependent Cl-channel

Fig.
5-14 to 5-17
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5-14 to 5-17
5-14 & 5-15
5-16
CHAPTER 6: Conclusion and Future Work

Section 6.1 – Conclusion

The photoreceptors in the distal retina convert light quanta into electrical signals that are transduced through 2nd order neurons (bipolar cells, horizontal cells, amacrine cells, ganglion cells) and transmitted via the optic nerve to the brain. Although the RPE is not directly involved in this process, its close anatomical relationship to the photoreceptors is fundamentally important for maintaining photoreceptor health and photoexcitability.

Vision is an energy (ATP) expensive process. Our photoreceptors expend large amounts of glucose, which is completely oxidized into CO$_2$ and H$_2$O (oxidative metabolism), or converted into lactic acid (glycolysis). Despite the extremely high choroidal blood flow, oxygen supply to the photoreceptors is limited; only $\approx$ 50% of all ATP used by the photoreceptors derive from oxidative metabolism. Therefore, large amounts of CO$_2$, lactic acid, and H$_2$O are released into the SRS, and their release almost doubles in the dark (see section 3-11). The accumulation of these metabolic waste products in the SRS would quickly destroy the photoreceptors, but the RPE protects the photoreceptors by transporting these metabolic waste products to the choroid.

Our *in vitro* experiments were designed to study CO$_2$ and lactate-transport mechanisms separately, but *in vivo* these mechanisms functionally interact with one another to enhance metabolic acid transport across the RPE. We elucidated the cellular mechanisms involved in CO$_2$ transport across the RPE, and showed that this process drives Na, Cl, HCO$_3$, and osmotically obliged water across the tissue, thus maintaining retinal adhesion to the RPE (section 3.9). We show that H$^+$-coupled lactate transport across the apical
membrane via MCT1 activates pH$_i$-regulatory mechanisms (section 5.2): (1) MCT1 activity stimulates Na/H exchanger (NHE); (2) MCT1 activity stimulates Na$^+$-linked HCO$_3$ co-transporters (NBC1 and NBC3), and this process is dependent on CA II activity. In the dark, these mechanisms help buffer both CO$_2$ and lactate induced cell acidification. Activation of these mechanisms increases [Na$^+$]$_i$, [HCO$_3$]$_i$, [lactate]$_i$, which drives isoosmotically obliged fluid across the apical membrane to cause cell swelling (section 4.6). Furthermore, the two-fold increase in CO$_2$ release by photoreceptors into the SRS causes its accumulation in the cell due to a $\approx$ 10-fold higher apical to basolateral membrane surface area (sections 3.2 & 3.11). This drives CA II-mediated CO$_2$ hydration into HCO$_3$, thus increasing [HCO$_3$]$_i$ further. Collectively, increased release of CO$_2$ and lactic acid by the photoreceptors causes cell swelling and osmotic stress. We show that the RPE reduces cell-swelling by activating Kir 7.1 K-channel at the apical membrane (section 4.5) and ClC-2 Cl-channel at the basolateral membrane (section 4.4). Furthermore, lactate efflux via AE2 at the basolateral membrane stimulates Ca$^{2+}$-activated Cl-channels (section 5.3), which drives fluid out of the basolateral membrane to help reduce RPE cell volume. KCl efflux via these channels mitigates CO$_2$- and lactate-induced RPE cell swelling, thus protecting the health of the RPE. We demonstrated how the RPE utilizes the changes in SRS CO$_2$ and lactic acid level as a signal to activate mechanisms that helps the RPE adapt to light-dark transitions in photoreceptor metabolism. Understanding these mechanisms provide us with the basis to understand the pathophysiology of disease (e.g., retinal edema and retinal detachment), which is a first step for the development of therapeutic interventions to preserve vision.
Fig. 6-1: Summary of conclusions.
Section 6.2 – RPE cell culture models for transplantation

As discussed in chapter 2 (section 2.1), the use of cell culture allows us to quickly and easily uncover physiological processes in the retinal pigment epithelium at a low cost. However, how closely does this in vitro culture model mimic the native tissue in vivo?

What are the differences in gene and protein expression, and physiology of fetal and adult RPE? Microarray data show that despite many overlapping genes between these tissues, many genes that are highly expressed in native RPE are lost in culture. Furthermore, genes that are normally not expressed in native RPE are upregulated in culture.

Improvement to the RPE cell culture model is a constant pursuit of the laboratory. The RPE culture model is used to study ion-transport mechanisms, map signal transduction pathways, study drug toxicity, and understand specific RPE functions. But in addition, it can also be developed for transplantation. RPE cultures that closely mimic native tissues can increase the success of transplantation in RPE-degenerative diseases such as AMD (Binder et al., 2004; Binder et al., 2007; da Cruz et al., 2007).

To improve RPE cell culture, we find clues in the environment of the RPE in vivo. What are the critical factors that influence RPE growth from its interactions with the retina and choroid? How can we mimic these conditions in vitro? For example, adding fibroblast conditioned medium to the basal side of chick RPE has been shown to improve tight junction development and expression, which facilitates RPE polarization and maturation (Rahner et al., 2004). What are the components of retinal extract used in RPE cell culture media (Hu & Bok, 2001) that enhances growth and maturation? Cultured hfRPE cells are grown in media containing 5% serum to promote cell proliferation and survival,
a condition that is not normally found \textit{in vivo} (Maminishkis et al., 2006). To this end, RPE monolayers cultured under serum-free condition has been developed by other laboratories (Gamm \textit{et al.}, 2008) and is better poised for transplantation purposes (serum may transfer animal pathogens to the patient) (Valtink & Engelmann, 2009). However, few experiments have been performed to study how closely this serum-free culture model mimics native RPE. Alternatively, embryonic and induced pluripotent stem cells (ES & iPS cells) have been coaxed to differentiate into RPE cells (Vugler \textit{et al.}, 2008; Carr \textit{et al.}, 2009a; Carr \textit{et al.}, 2009b). The transplantation of these cells into the eyes of mice and rats with retinal degeneration were able to rescue visual function. Based upon these findings, one may be interested in using fibroblast cells from patients, transform them into iPS cells, and subsequently convert them into RPE cells for transplantation into the same patient. This method is highly favorable because is eliminates the problem of immune rejection. Since vectorial transport of bicarbonate, lactate, and water by the RPE is critical to RPE-photoreceptor interactions, the feasibility of these various RPE cell culture models for transplantation can be evaluated by measuring the activities of these transport mechanisms.
Section 6.3 – Lactate, retinal detachment, and proliferative vitreoretinopathy

Separation of the retina from RPE induces many changes in retina/RPE morphology and physiology (Fisher & Lewis, 2003; Leiderman & Miller, 2009; Wickham & Charteris, 2009), for example: (1) non-neuronal glia cells (i.e., astrocytes, microglia, and Müller cells) in the retina undergo morphological changes and proliferation, (2) photoreceptors begin to degenerate – they become shorter and distorted, (3) horizontal and ganglion cells undergo extensive remodeling, (4) the RPE dedifferentiates and migrates into the subretinal space and retinal layers. These changes can begin 24 hours after retinal detachment and causes irreversible vision loss after three days if not treated. Furthermore, this condition can progress into proliferative vitreoretinopathy (PVR), a disease with poor visual prognosis characterized by inflammation, proliferation, and scarring (Pastor, 1998).

In PVR, RPE undergoes epithelial-to-mesenchymal transition (EMT), in which RPE loses its epithelial markers and morphology; transforming from cobblestone-shaped cells into elongated fibroblast-like cells (Casaroli-Marano et al., 1999; Saika et al., 2004). EMT of RPE cells in PVR involves transforming growth factor β (TGF-β) (Parapuram et al., 2009). In preliminary experiments, I excised cultured hfRPE monolayers grown on porous-filters, placed them on culture plates, and incubated them in cell culture media over 6 days (37 °C; 5% CO₂). In just three days, these RPE cells dedifferentiated into fibroblast-like cells via an EMT-like process (Fig. 6-2); they lose their pigmentation and cell-cell attachment, and undergo rapid proliferation and migration. It is likely that the hypoxic condition inherent in tissue cultures (due to large O₂ diffusion layer; see
discussion in section 4.2) activates EMT of RPE cells via hypoxia inducible factor 1 alpha (HIF-1α) (Higgins et al., 2007; Higgins et al., 2008; Brahimi-Horn & Pouyssegur, 2009). Although EMT of these cultured hfRPE cells are not induced by TGF-β, the downstream pathways and mechanisms activated by HIF-1α are mostly the same.

Fig. 6-2: RPE monolayers grown on porous filters undergo epithelial-to-mesenchymal transition. (A) 3 days (40x mag.), (B) 6 days (20x mag.), (C) 6 days (different sample; 20x mag.). (D) The
fibroblast-like cells were extracted and placed in a flask and grown (37 °C, 5% CO2) for 3 days (40x mag.).

In PVR, RPE cells undergo EMT and migrate into the SRS (Wickham & Charteris, 2009). Similarly, Müller glia cells dedifferentiate and migrate into the SRS (Lewis et al., 1999; Wickham & Charteris, 2009). Since cell division requires large amounts of energy, these dedifferentiated cells metabolize large amounts of glucose to produce significant amounts of CO2 and lactic acid. This is evidenced by the lower pH of the culture media bathing dedifferentiated RPE cells (Fig. 6-2 D) vs. that bathing confluent RPE cells (data not shown) – the pH difference was determined (subjectively) by comparing the difference in media color (i.e., dedifferentiated RPE media is noticeably more yellow than RPE media). Interestingly, lactate has been shown to stabilize HIF-1α by inhibiting HIF-1α prolyl hydroxylases (Crowther et al., 2001; Lu et al., 2002; Lu et al., 2005). Furthermore, recent studies also suggest that lactate upregulates HIF-1α in stem cells (Milovanova et al., 2008; Zieker et al., 2008). Since HIF-1α causes cell dedifferentiation (Jogi et al., 2002; Helczynska et al., 2003; Axelson et al., 2005), the large amount of lactate released by dedifferentiated RPE and retinal cells into the SRS may stimulate further RPE EMT in a positive feedback loop, thus exacerbating the PVR disease process. If true, targeting lactate transport mechanisms may be a viable strategy against PVR.
Section 6.4 – NBC3 activity, interactions, and function in RPE

The RPE expresses electrogenic (NBC1) and electroneutral (NBC3) NaHCO₃ co-transporters (NBC1) at its apical membrane (Fig. 1-8). NBC3 is an electroneutral Na/HCO₃ co-transporter, thus its activity is not limited by the membrane voltage; it is capable of transporting large amounts of NaHCO₃ and may play a significant role in RPE fluid transport. The importance of NBC3 to HCO₃- and fluid- transport can be understood by comparing NBC3 localization in RPE vs. CPE (choroid plexus epithelium) (see section 3.9). NBC3 is localized to the basolateral membrane in CPE, which secretes HCO₃ and fluid. On the other hand, NBC3 is localized to the apical membrane in RPE, which absorbs HCO₃ and fluid. This difference in HCO₃ transport-direction of RPE and CPE may be in part due to their difference in NBC3 membrane localization.

Interestingly, in mice, loss of NBC3 expression leads to retinal degeneration because NBC3-mediated HCO₃-transport helps buffer photoreceptor-generated metabolic acids (Bok et al., 2003). In chapter 5 (section 5.2), we demonstrated that MCT1 interacts with NBC1 to enhance both lactic acid and HCO₃ transport, and that this process depends on cytosolic CA (CA II). The same acid-base coupling mechanism between MCT1 and NBC3 may exist, which implicates NBC3 in lactate transport. However, physiological experiments to study this functional interaction were difficult to interpret and are inconclusive. This is in part due to the lack of specific (or non-specific) inhibitors against NBC3. In addition, its electroneutrality makes its activity “invisible” to detection by electrophysiological methods. In this work, all evidences for NBC3 activity in RPE are indirect (sections 3.3 & 5.2). Another way to study this transporter in RPE is via siRNA
knockdown technology, but the development of a specific siRNA against NBC3 is tedious and difficult. Alternatively, instead of working with RPE culture models, it is of considerable interest to study HCO$_3$ and fluid transport in the RPE and CPE of NBC3 knockout mice.
References


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