UNDERSTANDING THE FUNCTION OF BESTROPHIN

by

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SIGNED: Tyson R. Kinnick
I would like to acknowledge Alan Marmorstein as having been my advisor, and I thank him for the opportunity to earn my Ph.D. degree.

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Thanks Grandpa, Mom, Dave, Halli, Dad, Don, Stephen, Brian, Dean & Denise, Brian, Brent, Lena, Will & Dixie, and Kendra – all my love.
DEDICATION

I dedicate this dissertation to two individuals, my wife and my best friend.

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ABSTRACT

The purpose of this study was to investigate the function of the protein bestrophin-1 (best-1). Previous studies have suggested that best-1 is either a Ca\(^{++}\)-activated Cl\(^-\) channel (Cl\(_{Ca}\)) and/or that it is a regulator of Ca\(^{++}\) induced responses in the retinal pigment epithelium (RPE). My hypothesis is that best-1 regulates Ca\(^{++}\) homeostasis in the RPE.

To test this hypothesis, electrophysiology experiments were performed on whole monolayers of cultured primary fetal human retinal pigment epithelium (fhRPE). These cells were necessary for my work because they endogenously express best-1, they generate a sustained transepithelial resistance (TER) of greater than 1000 Ω*cm\(^2\), and I can modulate protein expression in them using replication defective adenoviral vectors.

The amount and polarity of the transduced proteins exposed at the cell surface was not altered by overexpression of best-1 or the best-1 mutants W93C or R218C. Best-1 (Wt) transduction of fhRPE increased baseline short circuit current (I\(_{sc}\)) over Controls and best-1 mutant experimental groups. Cl-ion substitution in the Wt group caused a reduction in I\(_{sc}\) that was reversible upon re-introduction of Cl\(^-\) to the bathing solution. Cl-ion substitution did not significantly alter I\(_{sc}\) in any other experimental group. With cell surface expression of best-1 and best-1 mutants the same, cell polarity of best-1 and best-1 mutants the same, and equivalent TER increases in response to Cl-ion substitution the same across all experimental groups, the data support a regulatory function for best-1 for Cl\(^-\) transport across the epithelium.
Application of the Ca\textsuperscript{++}-ionophore ionomycin resulted in a biphasic response in Control monolayers, which was reduced in monolayers overexpressing best-1. NFA (used to block Cl\textsubscript{Ca} currents) resulted in a decrease in the TEP of Controls and Wt monolayers to approximately half the value observed at the maximal response to ionomycin (Peak 2 or P2). The \( I_{sc} \) response of Control and Wt monolayers mimicked the TEP response, resulting in an \( I_{sc} \) at the end of exposure to NFA that was the same as the \( I_{sc} \) at Peak 1 (P1) of the biphasic response to ionomycin. R218C expressing cells showed little response to Ca\textsuperscript{++} and NFA, and W93C cells were not responsive.
CHAPTER 1

INTRODUCTION

The purpose of this study was to investigate the function of best-1 and how disruption of that function results in macular disease. By studying a monogenetic inherited eye disease such as BMD, I hope to be able to assist in determining the molecular processes involved in creating the macular degeneration and apply this knowledge to develop therapies for the multi-genetic macular degenerative disease known as age-related macular degeneration (AMD) – the leading cause of blindness in the Western world (Leibowitz et al., 1980). Currently, about 2 million citizens of the United States have AMD, with that number estimated to grow to 3 million by 2020 (The Eye Diseases Prevalence Research Group, 2004).

The results of this study support a regulatory role for best-1 in Ca\(^{++}\) homeostasis in the RPE, and support a loss of responsiveness to Ca\(^{++}\) stimulation for fhRPE overexpressing best-1 and the best-1 mutants W93C and R218C.

EXPLANATION OF THE PROBLEM AND ITS CONTEXT

The first manuscript (APPENDIX A) is a brief review of the structure and function of best-1. Currently, the most accepted structure of best-1 is that proposed by Petruhkin et al., 1998, upon identification of the gene responsible for BMD (see REVIEW OF LITERATURE). Mutations in the gene **BEST1** are responsible for the development of three types of autosomal dominantly inherited macular disease: BMD,
adult vitelliform macular dystrophy (AVMD) (Allikments et al., 1999; Seddon et al., 2001; White et al., 2000), and vitreoretinochoroidopathy (ADVIRC) (Yardley et al., 2004). The proposed structure of best-1 has four transmembrane domains and places the amino- and carboxy-termini in the cytoplasm. Other studies have supported this four transmembrane topology with the cytosolic termini using computer generated algorithms (Bakall et al., 1999; Hoppe et al., 2001). The first study to look at the function of best-1 used this four transmembrane model (Sun et al., 2002). In 2003, one study using three different methods: 1) insertional cysteine modification, 2) insertional N-linked glycosylation sites, and 3) insertional tobacco etch virus protease (TEVP) cleavage sites (Faber et al., 2001), proposed a model of best-1 with four transmembrane domains and a fifth membrane domain situated horizontally in the outer leaflet of the plasma membrane; with both termini located in the cytoplasm (Tsunenari et al, 2003). Weber and colleagues have proposed a model for best-1 with five putative transmembrane domains containing a cytosolic amino-terminus and an extracellularly located carboxy-terminus (White et al., 2000), but have more recently revised their model to four transmembrane domains (Milenkovic et al., in press). Another study, using two bestrophins cloned from *Xenopus laevis* oocytes and the SOSUI computer algorithm (Hirokawa et al., 1998), predicted a model with six transmembrane domains and termini located in the cytoplasm (Qu et al., 2003). Recently, a study using the well established *in vitro* translation/translocation *E. coli* leader peptidase (Lep) signal gene assay (Gafvelin et al., 1997; Johansson et al., 1993; Nilsson and von Heijne, 1993) has shown that best-1 is more likely a four transmembrane domain protein (Milenkovic et al., in press).
In addition to the tertiary structure of best-1, the determined quaternary structure differs based on the methods of study. Sun et al., 2002, used an indirect method to try to determine the quaternary structure of best-1. Two different epitope tagged plasmids of human best-1 (hbest-1) were co-transfected into human embryonic kidney-293 (HEK-293) cells at differing ratios, then labeled with $[^{35}\text{S}]$methionine, followed by immunoprecipitation, and Western blotting. It was determined that hbest-1 has a stoichiometry of 4 or 5 subunits per complex. With radiolabelling as the quantitative measure, there was no direct assessment of how many subunits were actually combined, as could have been determined by Western blot prior to the $[^{35}\text{S}]$methionine labeling experiments. It is important to note that the methods of Sun et al., 2002, do not take into account the possibility of protein aggregates based on improper folding. Using gel exclusion chromatography from tissue derived best-1 and velocity sedimentation (Clarke and Smigel, 1989), a dimeric protein-detergent complex was calculated to be 206 kDa, with the protein component estimated to be $\sim$138 kDa. Best-1 has a molecular weight of 68 kDa – leading to the homodimer conclusion for best-1 in porcine RPE (Stanton et al., 2006).

Presently, two hypotheses prevail for the function of best-1: 1) Best-1 is a Cl$_{Ca}$ that is responsible for generating the light peak (LP) of the electrooculogram (EOG)(see REVIEW OF LITERATURE), and disease causing mutations result in a loss-of-function (Chien et al., 2006; Sun et al, 2002; Tsunenari et al., 2003; Tsunenari et al., 2006; Qu and Hartzell, 2004; Qu et al., 2003; Qu et al., 2004). 2) Best-1 is not the Cl$_{Ca}$ that generates the LP (Marmorstein et al., 2006) and best-1 normally inhibits the LP by maintaining
RPE Ca\textsuperscript{++} homeostasis, most likely involving L-type voltage-dependent Ca\textsuperscript{++} channels (VDCCs) (APPENDIX B). Background for these hypotheses is written in the REVIEW OF LITERATURE. The first manuscript was written to provide a synopsis covering both hypotheses, and to review the known mutations in BEST1 (the gene encoding best-1) that have been identified in three maculopathies.

Chapter 2 of this dissertation is devoted to describing the fhRPE cell culture method established by the author in the laboratory of Dr. Alan D. Marmorstein. This culture method is that of Hu and Bok, 2000. However, since I established and maintained the cultures in the Marmorstein laboratory, I am including Chapter 2 as a subset of data to the field. These fhRPE cells were absolutely essential to most of my dissertation experiments for three reasons. First, they are the only RPE cells that endogenously express best-1. Second, monolayers of fhRPE produce TERs greater than 1000 Ω*cm\textsuperscript{2}. Third, best-1 expression in fhRPE can be modulated via transfection with replication defective adenovirus vectors to overexpress wild-type best-1 and best-1 mutants. These three parameters: the endogenous expression of best-1, the TER values greater than 1000 Ω*cm\textsuperscript{2}, and modulation of best-1 expression are important for the following reasons. Endogenous expression of best-1 in cultures of fhRPE is the closest thing to in vivo RPE available. In addition, high TER values, as discussed further in Chapter 2, are essential for recording changes in TER elicited by various experimental conditions. Tight-junctions form semi-permeable gaskets between RPE cells. If the tight-junctions are leaky, then electric current will flow paracellularly (through the tight-junctions and around the cells) instead of transcellularly (across, or through, the RPE cells) (Fig. 2A and
Fig. 5). If electric current flows paracellularly, then conductance changes across the RPE monolayer cannot be recorded. Furthermore, modulation of the expression of best-1 and best-1 mutants in this culture method allowed me to observe how the overexpression of these proteins affected the electrical properties of the cells, and also how the cells responded to different experimental conditions.

An equivalent electrical circuit representing RPE cells is shown in Figure 2B. The apical and basolateral membranes are represented by apical ($V'_{ap}$) and basolateral ($V'_{ba}$) potentials (generated by the polarized expression of ion conducting channels and transporters) in series with the resistors $R_{ap}$ and $R_{ba}$ representing the resistances of the apical and basolateral plasma membranes. A shunt resistance ($R_s$) is in parallel with $R_{ap}$ and $R_{ba}$, and represents the tight-junction complex resistance. The currents operating at the apical and basolateral plasma membranes at any given time represent a net current within the RPE cells called the short circuit current ($I_{sc}$). This current is what generates the potentials across the plasma membranes apically and basolaterally. It is important to note that in my dissertation work I never measured the apical and basolateral membrane potentials, because intracellular recordings are required for those measurements. As presented in greater detail in Chapter 4, the principle governing the resistance and $I_{sc}$ calculations performed in my work is Ohm’s law:

$$V(\text{voltage}) = I(\text{current}) \times R(\text{resistance}).$$

All functional studies of best-1 to date have used cell lines that do not endogenously express best-1. Most of these cell lines are not RPE cells and are not epithelial in character (like HEK-293 cells). If I want to investigate the function of best-1
in RPE cells, then I need a method in which I can do this. These cell lines also do not produce TERs above a background of \(\sim 130 \Omega \text{cm}^2\). The relationship between best-1 expression and TER development is not known. HEK-293 cells have been used the most in studying best-1 function via whole-cell patch-clamp. Unfortunately, no cell line exists outside of a Petri dish, and HEK-293 cells are derived from kidney and not eye cells. Primary cultures of fhRPE possess many of the characteristics of RPE in the eye, including endogenous expression of best-1.

Chapter 3 is presented as it has been prepared for submission as a letter to the editor for Experimental Eye Research. This work examined the role of the melanosome in forming lipofuscin in RPE cells. Lipofuscin accumulates in RPE cells naturally with age, but its rate of accumulation in some maculopathies (Best disease, Stargardt’s disease) is accelerated. Lipofuscin is believed to result, at least in part, from the incomplete degradation of phagocytosed photoreceptor outer segments (OS) (Eldred, 1989; Eldred, 1995; Hogan, 1972). Lipofuscin accumulation resulting from incomplete degradation of phagocytosed OS is supported by the finding that the major component of lipofuscin, the bis-retinoid pyridinium salt \(N\)-retinylidene-\(N\)-retinylethanolamine (A2E), is derived from OS (Fishka et al., 2005; Mata et al., 2000; Sparrow et al., 2000). My work, presented in Chapter 3, investigated the role of the melanosome in degrading phagocytosed OS. My work is significant because for the first time a primary cell culture method (fhRPE) has been used to study the role of the melanosome in OS degradation in the RPE. Use of the fhRPE primary cultures allows for the modulation of expressed proteins in the RPE cells using replication defective adenoviral vectors. My contribution
to the study was the establishment and maintenance of the fhRPE cells, modification of existing protocols for the isolation of human and porcine OS, organizing and setting up the experiments, and performing the pilot study and successive experiments.

The second manuscript (APPENDIX B) examined the effect of best-1 and best-1 mutant overexpression in a rat derived RPE cell line (RPE-J), and also showed *in vivo* that the L-type Ca$$^{++}$$ channel blocker, nimodipine, reduced the LP in the rat DC-ERG (see REVIEW OF LITERATURE) with no effect on the a- and b-waves. In RPE-J, overexpression of best-1 resulted in a shift in the voltage-dependent activation of L-type Ca$$^{++}$$ channels to more negative values, closer to the resting potential of RPE, and accelerated the activation kinetics of the channels. The W93C mutant created a decrease in the activation and inactivation kinetics of L-type Ca$$^{++}$$ channels while R218C accelerated the activation and inactivation kinetics. This second manuscript is significant because it showed for the first time that best-1 and best-1 mutants affect L-type Ca$$^{++}$$ channel kinetics. For the most part this work preceded, and inspired to a great degree, the work of the present study through collaboration with the laboratory of Dr. Olaf Strauss, Neal Peachey, and Lihua Marmorstein. My contribution was the cell surface protein biotinylation work presented in Figure 3A.
GENERAL STUDY AIMS

AIM 1: To investigate differences between fhRPE cells, adult human RPE cells, and established human and rat derived RPE cell lines.

AIM 2: To investigate best-1’s contribution to RPE Cl\(^-\) transport, and to investigate the effect of best-1 mutant proteins W93C and R218C on RPE Cl\(^-\) transport.

AIM 3: To investigate the effects of best-1 and the best-1 mutants W93C and R218C on the RPE’s response to a Ca\(^{++}\) stimulus.

REVIEW OF LITERATURE

The macula

The macula of the eye is a circular area of the neural retina, approximately 5 mm in diameter, located temporal to the optic disk and corresponding to the posterior pole of the eye, directly opposite the pupil and lens (Fig. 1A and 1B). The macula provides central, high-acuity color vision. At the center of the macula is the central foveola which contains only cone photoreceptors (Fig. 1C and 1D). This high-acuity vision allows people the ability to perform daily tasks like reading, and facial and object recognition. In general, when vision is referred to in conversation, it is the central vision provided by the macula. Damage to the macula almost always results in a decrease in vision.

The neural retina and the RPE

The retina consists of ten layers (listed from most interior outward)(Fig. 3):

1) Membrana limitans interna (inner limiting membrane); established by Müller cells (glial satellite cells)
2) Layer of nerve fibers (stratum opticum); nerve fiber layer (nfl)
3) Ganglionic layer, consisting of nerve cells; ganglion cell layer (gcl)
4) Inner molecular, or plexiform, layer
5) Inner nuclear layer, or layer of inner granules; containing the soma of bipolar
cells, horizontal cells, and amacrine cells
6) Outer molecular, or plexiform, layer
7) Outer nuclear layer, or layer of outer granules; containing the soma of rod
and/or cone photoreceptors
8) Membrana limitans externa; established by Müller cells
9) Layer of rods and cones; containing the inner and outer segments of the
photoreceptors

10) Pigmentary layer (tapetum nigrum) – the retinal pigment epithelium
(RPE)

As listed, the first nine layers are commonly referred to as the neurosensory retina
because they contain all of the nerve cells of the eye responsible for vision. The tenth
layer, the RPE, is intimately related to the distal portion of the neural retina – the OS of
the photoreceptors interdigitate with the apical microvilli of the RPE (Fig. 1D, and
Fig. 3). Superficial (more outward) to the RPE is the choroid of the eye containing a
vascular bed that is separated from the neural retina by the RPE. The inner retina is
vascularized to within, but not beyond, the inner nuclear layer. Beginning at the margins
of the macula, these vessels decrease until there are none at the foveola (the most central
region of the macula)(Fig. 1B and 1D). Superficial to the choroid is the sclera, or the
“white of the eye” (that portion of the eye one can see when looking into the face of another person). The RPE functions are many, one of the most important is its physical formation of the blood-retinal barrier serving to separate and protect the neural retina from blood vessels and blood cells that would diminish the quality of vision. The RPE cells also vectorally transport ions and metabolites from the subretinal space (between the neural retina and the RPE) to the blood in the vascular bed of the choroid, and vectorally transport nutrients to the outer retina from the blood. When there is a dysfunction in the relationship between the photoreceptors of the neural retina and the RPE, resulting in the death of the photoreceptors of the macula – high acuity color vision (the vision used for reading, and facial and object recognition) is lost. This is called macular degeneration, and presently there are no therapies to remedy loss of vision by any of the unknown mechanisms that cause macular degeneration (refer to the first paragraph of the INTRODUCTION to Chapter 1, and “The Macula” under the REVIEW OF LITERATURE). This is why the RPE is so critical to the health of vision, and why the RPE is important to my research.

**Best vitelliform macular dystrophy**

The first documented cases of BMD were described in 1905, by Best, as a form of juvenile vitelliform macular degeneration. Best disease is an autosomal dominant inherited macular degeneration. The term vitelliform comes from the characteristic “egg-yolk-like” lesion found in the macular region upon fundus examination (Cross and Bard, 1974; Gass, 1997; and Marmor, 1979). The *VMD2* gene, encoding the protein best-1, was identified in 1998 (Petruhkin et al., 1998; Marquardt et al., 1998).
It is imperative to note that in December 2006, the official nomenclature for the \textit{VMD2} gene, and the three human related paralogues was changed. \textit{VMD2} is now \textit{BEST1} (best-1 gene), vitelliform macular dystrophy-2-like-1 (\textit{VMD2L1}) is now \textit{BEST2} (best-2 gene), \textit{VMD2L3} is now \textit{BEST3} (best-3 gene), and \textit{VMD2L2} is now \textit{BEST4} (best-4 gene). Hence, \textit{BEST1} will be used from this point on in the dissertation when referring to the gene, and best-1 will continue to be used to refer to the protein.]

Petruhkin et al., 1998, had analyzed a large Swedish family with individuals in Älvdalen (Barkman, 1961) and Vilhelmina (Nordstrom et al., 1972). This family was traced back 12 generations, with the earliest ancestral couple born in the 1670s. Subsequent epidemiological studies have suggested that families in Minnesota carrying the BMD disease are relatives to Swedish immigrants (Nordstrom, 1980). The prevalence of BMD has been estimated to be 2 per 10,000 in the Swedish population. Three hundred Israeli citizens have been diagnosed with Best disease, and in the United States approximately 14,000 people have Best disease (The Association For Fighting Best Disease, Israel) (http://www.best.org.il/)

To date, 100 mutations in the \textit{BEST1} gene have been identified (http://www.huge.uni-regensburg.de/VMD2_database/index.php?select_db=VMD2). All known mutations are autosomal dominant in inheritance with most being missense mutations causing BMD; however, four mutations are associated with adult vitelliform macular dystrophy (AVMD)(Allikmets et al., 1999; White et al., 2000; Seddon et al.,
2001), one mutation results in “bulls-eye” maculopathy (Allikmets et al., 1999), and three other mutations cause autosomal dominant vitreoretinochoroidopathy (ADVIRC) (Yardley et al., 2004) (see APPENDIX A). Except for one truncation mutation found in the carboxy terminus of a single patient, all other best-1 disease causing mutations are located in the amino terminal 364 residues of the protein that contain the conserved putative transmembrane domains. Most mutations are within, or close to, the putative transmembrane spanning domains.

BMD has typically been diagnosed based on a fundus examination and an EOG, but now genetic testing is available and the results are definitive. As a result of genetic testing, EOGs are no longer performed for diagnosis of BMD at institutions where genetic testing is available because the results are obtained more quickly and definitively for the same cost as the older EOG test. BMD is usually diagnosed between the ages of 3-15 years old. Historically, BMD has been thought of as a juvenile onset maculopathy (Godel et al., 1986), but most patients today don’t present with any vision loss (resulting in diagnosis) until the 4th or 5th decades of life. My opinion is that even 50 to 60 years ago people were not living as long as they do today, and as a result of that reason not many people lived long enough to be diagnosed with BMD. BMD patients will present with a yellow subretinal blister at the macula during a fundus examination. In spite of the subretinal blister, some patients have normal vision. Vision loss at diagnosis, and the rate of vision loss with disease progression varies among BMD patients, with some patients maintaining good vision throughout life.
The progression of BMD has been categorized into stages. These stages have
been defined by Gass, 1997, and are outlined as follows:

- **Stage 1 (previtelliform):** Normal macula or subtle RPE pigment changes; EOG abnormal; visual acuity normal.
- **Stage 2 (vitelliform):** Well-circumscribed, 0.5-5 mm round, elevated, yellow or orange lesion; described as an “egg-yolk” appearance; usually centered on the fovea; can be multifocal; the rest of the fundus has a normal appearance; visual acuity 20/20 – 20/50.
- **Stage 3 (pseudohypopyon):** Yellow material can accumulate in the subretinal space, in a fluid filled cyst. The fluid will shift with extended changes in position (60-90 minutes). Visual acuity 20/20 – 20/50.
- **Stage 4 (vitelliruptive):** Scrambled-egg appearance is due to the breakup of the uniform vitelliform lesion. Pigment clumping and early atrophic changes may be noted. Visual acuity may deteriorate moderately. Visual acuity 20/20 – 20/100.
- **Stage 5 (atrophic):** As the yellow blister dissipates with time, an area of RPE atrophy remains. This appearance is difficult to distinguish from other forms of macular degeneration. Visual acuity can deteriorate more markedly at this stage, and patients are often legally blind (20/200 or less).
Stage 6 (choroidal neovascularization): Following the atrophic stage, choroidal neovascularization can develop, leading to a whitish subretinal scar. Legal blindness is common at this stage.

The clinical ERG, the DC-ERG, and the EOG

The clinical electroretinogram (ERG), the direct-current ERG (DC-ERG), and the EOG all measure the standing potential across the eye, with the cornea positive. The ERG has been in use since the early 1900s. By the mid-1930s the components of the ERG were beginning to be differentiated, and in 1962, a method for recording the standing potential across the eye, with the cornea positive to the posterior of the eye was developed (Arden et al., 1962). This technique was the EOG (Fig. 4). Details of the EOG procedure are provided in the legend to Figure 4. An Arden ratio (light peak to dark trough ratio in the EOG) of less than 1.55 (together with a vitelliform lesion, family history, and a normal clinical ERG) is clinically diagnostic for having BMD (Cross and Bard, 1974). Cross and Bard set the diagnostic cut-off for BMD at an Arden ratio of 1.55 because this was an Arden ratio value that separated graphically the EOG Arden ratios for known BMD patients from known non-BMD patients (normal controls). A reduced LP, as observed in an EOG test, used to be the only fully penetrant sign of BMD, until the development of genetic testing (see previous section).

Research at the cellular level has shown that the LP is generated by a Cl− conductance across the basolateral plasma membrane of the RPE (Gallemore and Steinberg, 1989a,b). These studies used explants of chick retina-RPE-choroid to study the LP and effects on the LP caused by treatment with the anion conductance blocker
4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS). Explants were placed in an Ussing chamber, providing independent access to both the apical and basolateral plasma membranes. DIDS (10 – 50 µM) applied basolaterally reduced the LP ~40%. This effect was reversible. In additional experiments where glass recording electrodes were placed in the subretinal space, within an RPE cell, and in the subchoroidal space simultaneously during an experiment, Gallemore and Steinberg observed that DIDS (10 – 125 µM) caused a decrease in the TEP of the explant in a two phase effect -- 1) hyperpolarization of the basolateral membrane followed by 2) depolarization of the apical membrane. During DIDS treatment of the explants, the resistance of the basolateral plasma membrane increased, as did the intracellular Cl⁻ activity (as measured in additional experiments using Cl-selective microelectrodes). The hyperpolarization of the basolateral membrane, the depolarization of the apical membrane, the increase in basolateral membrane resistance, and the increase in intracellular Cl⁻-activity in response to treatment of the explant with DIDS, are all consistent with the inhibition of a Cl⁻ conductance across the basolateral plasma membrane of the RPE, and suggest that this conductance (as shown in the chick LP experiments) generates the LP. Figure 5 shows an image of some of the known transporters and ion channels of the RPE. By convention, the apical membrane of the RPE is set positive to the basolateral membrane in electrophysiology experiments [consistent with the corneofundal potential established in EOG recordings on eyes (Fig. 4)]. In 1996, Hu et al., performed Cl⁻ flux experiments whereby net unidirectional fluxes of \(^{36}\text{Cl}⁻\) were measured across fhRPE monolayers placed in an Ussing chamber. Hu et al., found that cultured fhRPE showed a net apical-
to-basolateral flux of Cl\(^-\). This is shown in Figure 4 as positive current (as described in the figure legend). This positive current is the current that generates the LP and can be recorded during an EOG test.

The clinical ERG is measured using alternating current over a short duration of time (~100-250 msec). The DC-ERG and EOG both use direct-current to measure the standing potential across the eye over a longer time duration (10 or more minutes). The EOG is performed on humans because it is more comfortable for the subject and has a more steady baseline compared to the inherent baseline drift of DC-recording. The EOG protocol does not place an electrode directly on the cornea of the eye, as requisite for DC-ERGs. Since the EOG takes a minimum of 30 minutes to perform following dark adaptation, an electrode placed on the cornea for that amount of time would become uncomfortable.

The ERG is comprised of two major components, the a- and b-waves. The a-wave (negative in deflection) reflects the light-induced decline in dark current around the rod OS (Lamb, 1996). The b-wave (positive in deflection) follows the a-wave and is produced by the activity of second-order retinal neurons (bipolar cells) receiving input from the photoreceptors (Hood and Birch, 1996; Kofuji at al., 2001; Robson and Frishman, 1995). Because these responses are short in duration (usually complete by 250 ms) they can be readily recorded using AC-coupled amplification at high sampling rates.

Following the a- and b-waves, there are three potential changes that can be recorded across the eye that are produced by the RPE in response to light and are only
observed in the DC-ERG and EOG. The high frequencies of the a- and b-waves cannot be easily detected (if at all) with the low frequency properties of the DC-amplifier. The first of these potentials is the c-wave (positive in deflection) which is generated in response to a decline in extracellular \([K^+]\) in the subretinal space from \(~5\) mM to \(~2\) mM in response to the light-induced closure of \(Na^+\) channels along the photoreceptor OS and is the sum of two potentials: a positive potential generated by the RPE and a negative potential generated by the Müller cells of the distal retina (known as slow PIII) (Witkovsky et al., 1975). The c-wave occurs within 10 seconds of recording and is immediately followed by the fast oscillation (FO)(negative in deflection). The FO represents the recovery of the potential of the eye to at or below the dark-adapted baseline and represents a hyperpolarization of the basal membrane of the RPE. The FO reaches a minimum level within the first two minutes following a light stimulus. The LP is a positive polarity response which follows the FO and reaches a maximum in humans at 5-9 minutes after light onset (Linsenmeier and Steinberg, 1982). The LP of the EOG is signaled by an unidentified light peak substance (LPS) that is believed to be secreted by the photoreceptors when exposed to light (Gallemore et al., 1998). The LP is produced by depolarization of the basolateral plasma membrane of the RPE (Gallemore and Steinberg, 1993; Gallemore et al., 1998; Griff and Steinberg, 1982; Griff and Steinberg, 1984; Linsenmeier and Steinberg, 1982; Linsenmeier and Steinberg, 1984; Steinberg et al., 1985). Studies have shown that the depolarization of the basolateral membrane is caused by activation of a Cl⁻ current (Gallemore and Steinberg, 1989a; Gallemore and Steinberg, 1989b; Gallemore and Steinberg, 1993; Gallemore et al., 1998), and this
current is Ca$$^{++}$$-sensitive (Burgess et al., 1997; Marmorstein et al., 2006; Rosenthal et al., 2006).

**Lipofuscin accumulation and the diminished LP**

Lipofuscin is a autofluorescent substance known to accumulate at an accelerated rate in the RPE of BMD patients (see EXPLANATION OF THE PROBLEM AND ITS CONTEXT and Chapter 3). There is a disconnect in trying to better understand the function of best-1 because the accumulation of lipofuscin and the diminished LP are, at present, separate signs of the same disease. The focus of the present study has been on understanding the function of best-1. In addition, I have used the fhRPE cultures to pilot a study to determine if the fhRPE cells and the protocols I have employed are valid for future experiments looking at the formation and accumulation of lipofuscin within the RPE. This work is presented in Chapter 3. Future experiments related to this work will involve overexpression of best-1 and the best-1 mutants W93C and R218C in fhRPE cells fed OS in the presences or absence of light to determine if, and how, best-1 and the mutants alter the accumulation of lipofuscin within fhRPE. I am hopeful that these future experiments will illuminate some relationships between the accumulation of lipofuscin and Ca$$^{++}$$ stimulated ion conductances in the fhRPE overexpressing disease causing mutants. Finding a connection between these two signs of the disease would greatly benefit the field of vision research by providing a basis for therapies for BMD that treat both defects simultaneously. Therapies developed to treat BMD might also be used to help treat age-related macular degeneration (AMD), the leading cause of non-corrective blindness in the Western world (Leibowitz et al., 1980).
**Best-1 structure, sequence, and localization**

Best-1 is a 585 amino acid protein with a mass of 68 kD and an isoelectric point of 6.5-6.9. It is predicted to have at least four transmembrane domains with intracellularly located amino and carboxy termini (Bakall et al., 1999; Milenkovic et al., *in press*; Petrukhin et al., 1998; Tsunenari et al., 2003)(see first paragraph of EXPLANATION OF THE PROBLEM AND ITS CONTEXT). Based on Northern blotting and *in situ* hybridization data (Marquardt et al., 1998; Petrukhin et al., 1998), mRNA for best-1 is predominantly found in the RPE, but message was also found in the brain and spinal cord (Petrukhin et al., 1998). Best-1 protein has only been found in RPE cells (Stanton et al., 2006) in the basolateral plasma membrane (Bakall et al., 2003; Marmorstein et al., 2000); and the protein has not been detected in the widely used human derived RPE cell lines D407 and ARPE-19, nor in the rat derived RPE cell line RPE-J (Marmorstein et al., 2000).

Best-1 is a member of the large RFP family of proteins, often called bestrophins, originally identified in *Caenorhabditis elegans*, named for the presence of a conserved arginine (R), phenylalanine (F), and proline (P) amino acid sequence motif. Currently, the RFP proteins are proposed to be ion channels or regulators of ion channels (APPENDIX B; Chien et al., 2006; Marmorstein et al., 2004; Marmorstein et al., 2006; Rosenthal et al., 2006; Sun et al, 2002; Tsunenari et al., 2003; Tsunenari et al., 2006; Qu and Hartzell, 2004; Qu et al., 2003; Qu et al., 2004). There are homologous bestrophins in many species of vertebrates and invertebrates (Milenkovic et al., 2006; Tsunenari et al., 2003), with the amino terminal portions (~364 amino acids) being conserved and the
carboxy termini varying. The human bestrophins are more like their homologues than their paralogues (Milenkovic et al., 2006; Tsunenari et al., 2003) in the amino terminus, with 82% amino acid (aa) identity between hbest-1 and mbest-1 (mouse), 95% aa identity between hbest-2 and mbest-2, 93% aa identity between hbest-3 and mbest-3, and 69% aa identity between hbest-4 and mbest-4 (Tsunenari et al., 2003).

**Best-1 function**

This section focuses on the electrical properties of the RPE that generate the LP (see Fig. 4 and Fig. 5), and how best-1 and best-1 mutant expression in cultured cells and in the RPE in vivo (using animal models) affects the electrical properties of the cells or the RPE. Going from performing electrophysiology (whole-cell patch-clamp) experiments to looking at alterations in the LP of knock-out (not expressing best-1) and knock-in (expressing mutant best-1 protein) animal models of Best disease has always lacked a connection between the two methods. In the present study (Chapter 4), an in vitro RPE model is used for the first time to study best-1 and best-1 disease causing mutants at the tissue level.

**Cellular Research**

Best-1’s location in the basolateral plasma membrane, coupled with data showing Ca\(^{++}\)-activated Cl\(^-\) currents in HEK-293 cells overexpressing best-1, and lack of Ca\(^{++}\)-activated Cl\(^-\) currents in HEK-293 overexpressing best-1 mutants, has led to the simplest hypothesis to explain the data – that best-1 is the Cl\(_{Ca}\) responsible for generating the LP, and that mutating best-1 results in a loss-of-function of the channel (Sun et al., 2002; Tsunenari et al., 2003; Qu et al., 2003; Qu et al., 2004; Qu and Hartzell, 2004). Some
data supporting this has shown that hbest-1 and hbest-4 in HEK-293 cells produce linear current-voltage (I-V) relationships with whole-cell patch-clamp, and that *C. elegans* best-1 (cebest-1) and *Drosophila* best-1 (dmbest-1) produce rectifying I-V relationships – inward for cebest-1 and outward for dmbest-1 (Sun et al., 2002). When experiments were performed on 11 control EGFP-transduced cells, no current was detected. In the same study, caged-Ca$$^{++}$$ experiments were performed. These experiments used Ca$$^{++}$$ bound to a chemical called o-nitrophenyl EGTA-K4 (NPEGTA)(Molecular Probes). Ca$$^{++}$$-NPEGTA dialyzed into HEK-293 cells via the recording pipette releases Ca$$^{++}$$ cytosolically with exposed to ultraviolet light (UV light). With this method the authors showed that there was no hbest-1 current prior to a flash of UV light, and that following the flash hbest-1 expressing cells produced a current at +80 mV of 100-500 pA. When the same experiments were performed in the presence of the Ca$$^{++}$$ chelators 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetate (BAPTA) or ethylenediaminetetraacetic acid (EGTA); hbest-1 generated currents were less than ~50 pA. Additional experiments performed in this study showed that hbest-1 and hbest-2 induced currents were 90% blocked by administration of 0.5 mM DIDS, a blocker of many anion channels (see second paragraph of *The clinical ERG, the DC-ERG, and the EOG* in the REVIEW OF LITERATURE, and Gallemore and Steinberg 1989a,b).

Additional support of the Cl$$^{Ca}$$ hypothesis comes from work where cysteine modification of hbest-1 (Tsunenari et al., 2003) and mbest-2 (Qu et al., 2004; Qu and Hartzell, 2004) at amino acid residues residing within the second putative transmembrane
domain (TMD-2) has resulted in altered permeability and conductance responses to anions of different size.

The hypothesis that bestrophins are Cl\textsubscript{Ca}s, is strengthened by data from excised-patch and single channel recordings. Two laboratories have attempted this in the past year. Tsunenari et al., 2006, overexpressed hbest-4 in two cell lines: Chinese hamster ovary-K1 (CHO-K1) cells and HEK-293 cells. In excised membrane patches from these cells, Cl\textsuperscript{-} currents were observed. The activation and deactivation kinetics of the data shown were slow (10 or more seconds), suggesting the involvement of a possible membrane-associated messenger (as stated by the authors).

A second group, Chien et al., 2006, recorded single channel Cl\textsuperscript{-} currents in *Drosophila* S2 cells. *Drosophila* S2 cells are derived from late embryonic stages of *Drosophila melanogaster* (fruitfly) and are formally known as *Drosophila melanogaster* Schneider line 2 (S2) cells. *Drosophila* S2 cells express an endogenous Cl\textsubscript{Ca} current. Chien et al., set out to investigate whether this current is due to bestrophins. Using RT-PCR, the message for four *Drosophila* bestrophins (dmbests) was found. Using RNA interference (RNAi) to knock down the message of the four dmbests observed, Cl\textsubscript{Ca} currents could be significantly reduced in S2 cells using RNAi to dmbest-1 and dmbest-2 but not for dmbest-3 or dmbest-4. It was concluded that dmbest-1 and dmbest-2 compose the Cl\textsubscript{Ca} current observed in S2 cells. To prove that the S2 currents recorded in the S2 cells were Cl\textsuperscript{-} channels, dmbest-1 and dmbest-2 were expressed in HEK-293 cells. Dmbest-2 expressed in HEK cells did not induce any Cl\textsuperscript{-} currents using the whole-cell patch-clamp recording configuration but dmbest-1 was found to be sufficient to produce
Cl\(^-\) currents in HEK cells. This data is consistent with previous HEK whole-cell patch-clamp work (Sun et al., 2002; Tsunenari et al., 2003; Tsunenari et al., 2006; Qu and Hartzell, 2004; Qu et al., 2003; Qu et al., 2004), but once again is studying overexpression of a bestrophin in a non-native, non-epithelial cell line. However, Chien et al., did anticipate this criticism and mutated an amino acid in dmbest-1 (F81C), homologous to amino acid (F80) in mouse bestrophin-2 (mbest2) that altered Cl\(^-\) conductance and permeability (Qu and Hartzell, 2004; Qu et al., 2006). When dmbest-1-F81C was overexpressed in HEK cells, the Cl\(^-\) current observed was not linear as for wild type (wt) dmbest-1 but was inwardly rectifying – suggesting that the observed induced Cl\(^-\) currents were indeed a result of dmbest-1 overexpression. Chien et al., also performed single-channel recordings of S2 Cl\(^-\) currents. The caveat is that the recordings did not work in the absence of 3 mM adenosine triphosphate (ATP). Therefore, this data cannot be compared to data from previous work because previous studies did not use ATP in their experiments. ATP has long been considered the most likely candidate for being the light peak substance (LPS) released by the neural retina to trigger the cascade of events leading to the development of the LP. Intuitively, use of ATP to induce the proposed dmbest1 Cl\(^-\) currents in S2 cells makes sense, but then why have all of the whole-cell patch-clamp studies using HEK-293 cells, and even the patch-clamp experiments performed in this study on S2 cells, resulted in observed Cl\(_{Ca}\) currents without the use of ATP in the bathing or pipette solutions. Furthermore, the authors of the study were not able to verify that the channels they were observing were dmbest-1 or the other dmbests. While the two studies I have just presented are supportive of the Cl\(^-\)
channel hypothesis, they have not definitively proven that bestrophins are indeed Cl\textsubscript{Ca} channels.

**Animal Model Research**

Other work showing that best-1 does not generate the LP, that best-1 regulates RPE Ca\textsuperscript{++} homeostasis, and that best-1 normally functions to inhibit the LP has developed from the collaboration between four laboratories (Marmorstein et al., 2006). In 2004, a study reported a model for BMD in rats (Marmorstein et al., 2004). In this work, Long-Evans rats were subretinally injected with replication defective adenovirus vectors, then subjected to DC-ERGs were recorded at different luminance intensities. The authors observed a desensitizing shift in the LP response at different luminance intensities when best-1 was overexpressed, but the LP amplitudes across the range of intensities was not altered. However, the mutants best-1-W93C and best-1-R218C both reduced the LP amplitude across the range of stimuli tested. Best-1-R218C rats retained the ability to respond to increasing luminance intensities, but the responses were diminished in amplitude compared to best-1 controls. Best-1-W93C, however, was less responsive to increasing luminance intensities. In 2006, it was shown that best-1 knockout mice (KO) produce LPs that are usually greater in amplitude, but at least equal in amplitude, to best-1 homozygote mice at different luminance intensities. In addition, RPE cells isolated and dissociated from these mice showed identical Cl\textsuperscript{-} current densities via whole-cell patch-clamp recordings (Marmorstein et al., 2006), but this does not exclude the possibility that other channels are not compensating for the absence of best-1. Therefore, it has been shown that best-1 is not necessary to generate the LP, and suggests that best-1 may
function normally to inhibit the LP. Other experiments from that study showed that
nimodipine, a specific inhibitor of L-type voltage-dependent Ca\textsuperscript{++} channels (VDCCs),
reduced the LP in best-1 mice by 50\% and reduced the LP by 60\% in best-1 KO mice, but
compensation by other ion channels cannot be ruled out in these experiments. In
addition, the LPs of lethargic mice (harboring a loss of function mutation in the $\beta_4$
subunit of VDCCs) were reduced in amplitude at all but one luminance intensity tested –
what was expected for the best-1 KO mouse under the Cl\textsuperscript{-} channel hypothesis. This work
suggests contribution from the $\beta_4$ subunit in generating the LP in response to intracellular
Ca\textsuperscript{++}. Additional details of the work supporting these hypotheses are presented in
APPENDIX B, in the present study (Chapter 4), and in Marmorstein et al., 2006. All of
this work provided the basis for my dissertation research.
Figure 1: The Macula

The macula is located in the peripheral region of the eye near the optic nerve (A). Viewed through an ophthalmoscope (B), the macula is seen as a region encircled by a “vascular arcade” at the center of which is the fovea which appears as an orange spot due to a high local concentration of the yellow pigments lutein and zeaxanthene. The histologic subdivisions of the macula are labeled. While the fovea and foveola can be discerned clinically, the borders of the parafovea and perifovea cannot. The distribution and density of rod and cone photoreceptors differs in the macula and the peripheral retina with the number of cones greatest in the fovea (C). The retina is organized into layers of specific cell types which differ due to excavation of inner retinal neurons within the foveola as shown in D. Abbreviations: GCL = ganglion cell layer, Ph = photoreceptors, RPE = retinal pigment epithelium. A and B adapted from images publicly available from the National Eye Institute, National Institutes of Health website (http://www.nei.nih.gov/photo/).

Figure is adapted from Marmorstein and Marmorstein, Trends Gen, (in press).

Figure 2: Simple schematic of the RPE

(A) Two RPE cells (representing the monolayer) showing tight junctions and the possible paracellular and transcellular flow of electric current. The TEP is presented as the potential recorded across the monolayer via 0.5M KCl recording bridges placed apically and basolaterally. Not shown is that the baths are separated in the Ussing chamber. (B) Same schematic as (A), with an insert showing the equivalent electrical circuit of the
RPE. This circuit is represented by two series resistors ($R_{ap}$ and $R_{ba}$) in parallel with a shunt resistor ($R_S$) (representing the resistance of the tight junction). $R_{ap} =$ apical resistance; $R_{ba} =$ basolateral resistance. $V'_{ap}$ and $V'_{ba}$ represent the potentials of the apical and basolateral membranes, produced by the populations of ion conducting channels and transporters that are differentially polarized. Together $V'_{ap}$ and $V'_{ba}$ represent the $I_{sc}$ operating within the RPE monolayer.

Figure 3: Histology of the Neural Retina, the RPE, and the Choroid

From most interior to most exterior: vitreous humor, membrana limitans interna, layer of nerve fibers (stratum opticum), ganglion cell layer, inner molecular (plexiform) layer, inner nuclear layer (inner granules), outer molecular (plexiform) layer, outer nuclear layer (outer granules), membrana limitans externa, layer of rods and cones (inner and outer segments), retinal pigment epithelium (RPE), choroid.

Figure 4: The Electrooculogram (EOG)

The eye is electrically polarized, with the cornea positive to the fundus (posterior of the eye globe). (A) Schematic showing the cornea-positive movement of the eyeball in relation to electrodes placed at the canthi (corners) of the eye. When the cornea of the eye is moved toward the negative electrode located on the nose, the potential of the eye increases. When the cornea of the eye is moved toward the positive electrode located at the temple of the head, the potential of the eye decreases. *This is reversed in the figure. (B) The EOG is performed following 30 minutes of dark adaptation, and is
performed for 15 minutes in the dark, followed by 15 minutes in the presence of light illumination using a Ganzfeld bowl illuminator. The amplitude of the potential across the eye is determined while the subject makes alternating eye movements from side-to-side when prompted by flashing neon lights located at the back of the illuminator during the last 15 seconds of each minute during the EOG test. The amplitude of the potential across the eye is plotted for every minute of the test. The maximum amplitude of the potential across the eye in light is known as the light peak (LP) and is compared to the average potential across the eye in the dark. This ratio between the LP and the average dark potential is known as the Arden ratio – which is approximately 2.0 in normal individuals.

*Figure is adapted from Arden et al., Lancet. June 2, 1962, pp 1104-1105.*
Figure 5: RPE Transport, TEP, and Positive Current Flow

Ion channels and transporters of the RPE. By convention the apical plasma membrane has been set positive to the basolateral plasma membrane – consistent with the corneofundal presentation of the eye in the electrooculogram (EOG) (see Figure 4). Positive current, by definition, flows in the direction of movement of positive charges. Therefore, positive current flow across the RPE flows from apical to basolateral in direction (vitreous to choroid). *Note that even though Cl` anions move in a net apical-to-basolateral direction in the RPE, the current they produce, as defined above, is considered positive current.

*Figure is adapted from Marmor and Wolfensberger, The Retinal Pigment Epithelium: Function and Disease, 1998, p 112.*
Figure 1.
Figure 2.
Figure 3.
Figure 4.

Current flow around the orbit is the basis of eye movement potential.

Change in trace

Amplitude of response

Amplitude of response

Standing Potential µV*

Time (mins)
Figure 5.
CHAPTER 2

FhRPE CELL CULTURE METHOD

INTRODUCTION

The PRESENT STUDY (Chapter 4) relied on primary fhRPE cultures grown on permeable supports. These fhRPE cells were essential to most of the author’s dissertation experiments for three reasons. First, they are the only RPE cells that endogenously express best-1. Second, monolayers of fhRPE produce TERs greater than 1000 $\Omega \cdot \text{cm}^2$. Third, best-1 expression in fhRPE can be modulated via transduction with replication defective adenovirus vectors to overexpress wild-type best-1 best-1 mutants (see Chapter 1: EXPLANATION OF THE PROBLEM AND ITS CONTEXT).

METHODS

The culture method for obtaining fhRPE was as described previously (Frambach et al., 1990; Hu and Bok, 2000). RPE cells were collected from eyes of human fetuses of 20-24 weeks’ gestation. The tenets of the Declaration of Helsinki were followed, and consent for donation of tissue was provided. In a sterile hood, the anterior portion of each donor eye was removed using a #11 scalpel to initiate a cut extending through the sclera of the eye at the limbus, followed by a circumferential incision at the limbic arcade. After flipping the globe over, iris scissors were used to amputate the optic nerve through the retina. The retina was then removed by gentle shaking of the globe. From the hole made by removal of the optic nerve, iris scissors were used to cut outward from
the hole to produce three sections of roughly equal size. The pieces were then transferred to a 60 mm Petri dish containing Ca\(^{++}\) - and Mg\(^{++}\)-free phosphate buffered saline (CMF-PBS). Jeweler’s forceps were used to peel the choroid and RPE from the sclera. Once the sclera was removed, the RPE was physically dissociated from the choroid in sheets. These sheets were collected via a pipette. Care was given to make sure that no neural retina, red blood cell, choroid, or sclera contamination was present. The RPE explants were placed into one 15 mL conical tube and spun in a table top centrifuge at 2000 rpm for 10 minutes. Following centrifugation, the CMF-PBS was poured off and the explants resuspended in 1 mL of low Ca\(^{++}\) (50 µM) Chee’s essential medium (CEM) replacement-medium (305-310 mOsm) containing 1% bovine retinal extract (BRE) and 1% FBS (Hu and Bok, 2000). This suspension was then added to 7 mL of the same low Ca\(^{++}\) CEM replacement medium already placed in a sterile 60 mm Petri dish.

Cells were incubated at 37°C and allowed to proliferate for at least ten days before removing floaters (cells proliferated into the medium) to be frozen down (-80°C)(4 x 10^6 cells per mL). Cells were acquired every four days for a total of six acquisitions from each pair of donor eyes harvested.

For all experiments, fhRPE cells were plated on laminin coated Millicell-HA filters 0.4 µm pore size (12-mm; Millipore, Bedford, MA) at (2.5 x 10^5) cells/insert in CEM replacement medium with 1.8 mM Ca\(^{++}\), 1% BRE, and 10% FBS for their first week in culture. After one week, the 10% FBS was reduced to 1% FBS. FhRPE monolayers were maintained for at least 8-weeks prior to use in biotinylation and electrophysiology experiments [an established standard for fhRPE, especially regarding
best-1 expression (Fig. 1E)] (Frambach et al., 1990; Hu and Bok, 2000; Hu et al., 1994).

TERs were measured weekly until the cells were used in an experiment. TER values were measured in CEM replacement medium using a chopstick electrode and a voltohmmeter (World Precision Intruments, New Haven, CT).

RESULTS

Typically the TER of the cultures increased over a period of days to weeks post-plating. Figure 1B shows this development over time for a representative group of inserts maintained in the same 12-well plate, and from the same donor. FhRPE cultures plated on permeable supports attained an average TER at 10 weeks in culture of $1300 \pm 534 \, \Omega \ast \text{cm}^2$ (mean ± SD; n=993 and includes all monolayers used in this study) without adjusting for background resistance. Background resistance was determined from blank filters and was typically ~$130 \, \Omega \ast \text{cm}^2$. Figure 1A presents data from a pilot study performed to determine the best gestational age for donor tissue. As a result of this study, we modified our procurement criteria for fetal eyes to include only donor eyes of 20-24 weeks’ gestation. All of the fHrPE plated on permeable supports, or on glass or tissue culture plastic, became pigmented within 30-days (Fig. 1C), and formed hexagonal monolayers (Fig. 1D). Rate of pigmentation and pigmentation pattern varied with each donor. All monolayers that we have tested by immunoprecipitation and Western blotting have expressed bestrophin-1 (Fig. 1E). We have been able to maintain fHrPE cells on permeable supports for up to a year, and it has been reported that fHrPE can be maintained on permeable supports for at least two years (Hu et al., 1994).
DISCUSSION

The primary fhRPE cultures established according to the method of Hu and Bok, 2000, exhibit properties that are essential to the studies that were proposed to comprise my dissertation. These properties are a sufficiently high TER to permit the observation of changes in transepithelial electrical properties that are due to changes in membrane potential and conductance, and the endogenous expression of best-1. Hu and Bok reported unadjusted TER values for fhRPE of $776 \pm 31 \Omega \text{cm}^2$ (n=10; 1 month) and $834 \pm 31 \Omega \text{cm}^2$ (n=9; 2 months). Our monolayers averaged $1300 \Omega \text{cm}^2$ in culture medium. We routinely cultured blank filters to provide a reference for background resistance and this value was typically $\sim 130 \Omega \text{cm}^2$. Correcting for the average background resistance using the $130 \Omega \text{cm}^2$ value, the average TER for our cultures was $\sim 1170 \Omega \text{cm}^2$. This exceeds that reported by Hu and Bok by at least $200 \Omega \text{cm}^2$. This value also exceeds that reported by Maminishkis et al., 2006, by at least $450 \Omega \text{cm}^2$ -- for fhRPE cultured using a different method. Similarly, Hu and Bok reported best-1 expression in fhRPE cultured for two months, and we found best-1 expression for cultures to be maximal at 2 months (Fig. 1E).

In summary, I have successfully established and characterized primary human RPE cultures. These cultures permit the physiological recording of transepithelial electrical properties (TEP and TER). The monolayers exhibit a high TER and produce melanin pigment granules – two properties of RPE in the eye which are not readily reconstituted by available RPE cell lines. Importantly, these cells reproducibly express endogenous best-1. This last characteristic is critical because no functional studies of
best-1 in a native epithelium have previously been conducted. These are the only cell cultures that have convincingly been shown to express endogenous best-1 protein. This was an important preliminary part of my dissertation work, and has provided me with a culture method to study the physiological properties of RPE cells that normally express best-1 and how they respond to various experimental conditions.
Figure 1. FhRPE characteristics.

A: Number of cells harvested from proliferating cultures of fhrPE. Cultures were from donor eyes received at the gestational weeks indicated (20-37), and from one postnatal pair of eyes (3 months). From this pilot study we modified our donor criteria so that we only procure eyes from donors of gestational weeks 20-24. B: A representative line graph showing the TER development in one 12-well plate from a single donor as a function of weeks after plating. C: A representative image of fhrPE cells plated on a Millicell-HA nitrocellulose support (Millipore, Bedford, MA). Cells were 8 weeks post-plating at image acquisition. D: FhRPE cells form pigmented hexagonal monolayers on Millicell-HA supports. (X600) E: Best-1 expression at 1-, 2-, and 3-months post-plating on supports.
Figure 1.
CHAPTER 3

FORMATION OF LIPOFUSCIN IN HUMAN FETAL RPE FOLLOWING PHAGOCYTOSIS OF PHOTORECEPTOR OUTER SEGMENTS

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Abstract

The accumulation of autofluorescent deposits within the retinal pigment epithelium (RPE), known as lipofuscin, is a normal part of aging in humans. Lipofuscin accumulation, however, is accelerated in many forms of macular degeneration. Using primary fetal human retinal pigment epithelial (fhRPE) cell cultures that are highly pigmented, we report that lipofuscin-like granules are created and increased in number in fhRPE fed photoreceptor outer segments (OS) for 10-14 days. Exposure to light for 12 hours a day increased the formation of lipofuscin independently from OS phagocytosis. In five of six experiments performed, a reduction in the number of melanosomes was observed in correlation with an increase in lipofuscin and melanolipofuscin formation. These results provide a new assay for the study of lipofuscin formation and the role of the melanosome in the degradation of OS.

Keywords: lipofuscin, macular degeneration, phagocytosis, photoreceptor outer segment, retinal pigment epithelium
Introduction

Lipofuscin normally accumulates within every cell of the body with age, and is known as “age pigment” (Siakotos and Armstrong, 1975). The accumulation of lipofuscin with age within the retinal pigment epithelial (RPE) cells of the eye has been studied (Boulton, 1991; Boulton et al., 1990; Dorey et al., 1989; Dorey et al., 1993; Feeney-Burns et al., 1980; Feeney-Burns et al., 1984; Hogan, 1972; Kitagawa et al., 1989; Weiter et al., 1986; Wing et al., 1978). An increased accumulation of lipofuscin, relative to age-matched control eyes, is known to occur in some maculopathies such as BMD, Stargardt’s disease, Stargardt-like macular dystrophy, and pattern dystrophy.

Accumulation of lipofuscin in the RPE is also likely to play a role in the pathogenesis of the multigenetic disorder called age-related macular degeneration (AMD) – the leading cause of irreversible blindness in the Western world, affecting nearly 30% of those over the age of 75 years (Leibowitz et al., 1980). The observation that a reduction in photoreceptor cell number occurred in the presence of increased lipofuscin content in the RPE led to the proposal that the increased accumulation of lipofuscin may occur prior to photoreceptor cell death (Dorey et al., 1989). Therefore, it has been suggested that the high volume of lipofuscin formation may interfere with RPE function, though it could equally well be a consequence rather than a cause of dysfunction.

Lipofuscin is a term generically applied to an autofluorescent, membrane-bound intracellular material that is widely distributed in post-mitotic cells of different organs of the body (Kennedy et al., 1995). Lipofuscin in the eye, however, differs in three ways from the lipofuscin found in neurons, cardiac myocytes, skeletal muscle cells, liver,
spleen, testes, etc. Lipofuscin in the eye differs from other lipofuscin found throughout the body in 1) electron density, 2) uniformity of granule size, and 3) composition. Lipofuscin in the RPE cells of the eye usually contain a core composed of electron dense melanin pigment surrounded by the less dense lipofuscin. Lipofuscin in other cells of the body is uniform in density and the granules of lipofuscin vary in size. Lipofuscin granules of the RPE are relatively uniform in size and are believed to result, at least in part, from the incomplete degradation of phagocytosed photoreceptor outer segments (OS)(Eldred, 1989; Eldred, 1995; Hogan, 1972). This is supported by the finding that a major component of lipofuscin, the \textit{bis}-retinoid pyridinium salt \textit{N}-retinyldene-\textit{N}-retinylethanolamine (A2E), is derived from shed OS (Fishka et al., 2005; Mata et al., 2000; Sparrow et al., 2000). Lipofuscin in the other organs of the body has not been shown to contain A2E. Other work has shown that melanin is incorporated in the phagolysosomal system of OS degradation and lipofuscin formation, and suggested that melanin is either modified in the process or degraded (Feeney 1978). The purpose of this study was to determine 1) if we could induce fetal human RPE to produce lipofuscin by feeding them OS, and 2) to determine if we could observe changes in melanosome number in the process of lipofuscin formation – indicating active involvement in the OS degradation pathway.

\textbf{Background}

Bairati and Orzalesi (1963) observed lamellated bodies within the RPE and hypothesized that these bodies resulted from the detaching of discs from the apex of the
rod photoreceptor outer segment which had been phagocytosed by the RPE for degradation. The second part of the hypothesis was that loss of outer segments (OS) at the apex of the rod photoreceptor would be balanced at the base of the segment by formation of new discs. Their hypotheses were supported by the work of Droz (1963). Using radio-labeled amino acids, Droz tracked the migration of the amino acids from the inner to the outer segment of rod photoreceptors in rats and mice. Young (1967) improved the technique of Droz, replicated the findings in rats and mice, and extended the work to frogs. Two years later, Young and Bok (1969) were the first to report that the RPE actively participates in the disposal of rod OS, and defined the inclusion bodies containing the OS as phagosomes. Phagosomes of ingested rod OS were found to contain acid phosphatase activity and their contents varied – reflecting the various stages of degradation of the OS (Ishikawa and Yamada, 1970). In aged rats, Ishikawa and Yamada corresponded that the lipofuscin-type granules observed were residual bodies remaining from the phagosome – from incomplete degradation of its contents.

In the rhesus monkey, each RPE cell is associated with, on average, approximately 38 rods (cones in the fovea) and is responsible for the phagocytosis of 2000-4000 OS daily. In 75 years, this is the overbearing task of phagocytosing 7.92 x 10^7 OS per RPE cell (Young, 1971). Defects in the breakdown and phagocytosis of OS by the RPE contributes to retinal disease (Eldred, 1989; Eldred, 1995; Hogan, 1972). This is observed in the RCS rat that is able to bind shed OS but not ingest them, resulting in photoreceptor cell death, and in humans and rats harboring mutations in
c-mer (a tyrosine kinase receptor) that results in the inability to bind shed OS and in photoreceptor cell death. The enzymes, the proteins, and the intracellular organelle interactions involved in the pathway of degrading phagocytosed OS have been studied (Dell’Angelica et al., 2000; Desjardins et al., 1994; Diment et al., 1995; Feeney, 1973; Feeney-Burns and Eldred, 1983; Futter, 2006; Hogan, 1972). Lynette Feeney (1978) suggested that melanin was incorporated in the phagolysosomal system of OS degradation and lipofuscin formation, and suggested that melanin was either modified in the process or degraded. Gold labeled OS (in rats) and phagocytosed latex beads (in bovine choroid-RPE explants) both interact with melanosomes (melanin pigment granules with a cortex of reactive enzymes) of varying maturity (Schraermeyer et al., 1999). Lipofuscin accumulation in a human RPE cell line passaged at least 4 times and fed OS has been reported (Boulton and Marshall, 1986; Boulton et al., 1989; Rakoczy et al., 1992). This human RPE cell line (derived from adult human RPE) does not contain melanosomes, therefore, the role of the melanosome in the degradation of phagocytosed OS cannot be studied in it. The objective of the current study was to determine if cultured fetal human retinal pigment epithelial (fhRPE) cells are an appropriate model for studying the involvement of the melanosome in phagocytosis and degradation of OS with the long term goal of studying the role of best-1 in those processes.
Results

FhRPE cells were cultured as described previously (Hu and Bok, 2000). FhRPE were cultured on Millicell-HA inserts (Millipore, Bedford, MA) for at least 8 weeks prior to use in experiments. Inserts for the “dark” groups were only exposed to artificial light during feeding. Inserts in the “light” groups were exposed to artificial light for 12 hours per day. OS from porcine eyes were prepared under dim red light by the sucrose gradient centrifugation method as described (Molday et al., 1987). Except for one 10-day experiment, all treated inserts were fed (5 x 10^7) OS/day. Control inserts were always fed when the treated inserts were provided OS. For sucrose sedimentation gradients, the gradients were made just prior to loading the sample. Immediately after loading, the fraction tubes were spun at 103,000 X g for 2 hours. Photos for the representative sucrose gradient were taken with a digital camera under artificial white light and ultraviolet light. Fractions were removed via pipetting – each fraction was transported to a separate microcentrifuge tube. Granules in each fraction were determined using a hemacytometer and visualized at (x400) with DIC and fluorescence light microscopy. Where observed, values are expressed as mean ± SD. Groups were compared using Student’s t-test -- two-tailed assuming equal variances. Differences were considered statistically significant when the p value was less than 0.05.

Figure 1

Discontinuous sucrose gradients were prepared as in Figure 1A, and granules accumulated at the indicated interfaces (Figure 1B). A representative gradient from a human donor eye is shown in Figure 2A. The fluorescent characteristic of lipofuscin is
observed within the fractions of the gradient (Figure 2B). The amount of fluorescence decreased from fractions #1 through #3, as expected. Because each fraction was not re-run on additional gradients, there is some lipofuscin contamination in fraction #3 (Figure 2B).

**Figure 2**

In half of the experiments performed in the dark, lipofuscin formation increased at least 4.5-fold in the fhRPE cells fed (5 x 10^7) OS/insert/day, and melanolipofuscin (fraction #2) was created (Table 1). In the other three experiments, including the 10-day (1 x 10^8) OS/insert/day experiment and both 14-day experiments, the increase in lipofuscin formation in the OS treated fhRPE cells ranged from 1.4 to 3-fold. Melanolipofuscin formation also increased by 3.7-fold (10-day), 24-fold (14-day, Experiment I), and 2-fold (14-day, Experiment II). The general trend for all of the fhRPE cells maintained in the dark, except for the first 14-day experiment, was a decrease in the number of melanosomes in the OS fed cells compared to fhRPE not fed OS. Melanosome number for the “light” group in Experiment I was half the value of the melanosome number for the “dark” group, regardless of OS feeding. Experiment II exhibited the same number of melanosomes in the -OS and +OS fed groups exposed to light, while the melanosome number for the +OS group was half that of the -OS group maintained in the dark. Lipofuscin formation increased in the fhRPE cells when exposed to 12 hours of light per day without OS feeding (compare Fraction #1 in the -OS “light” group with Fraction #1 in the -OS “dark” group in Experiment I & II in Table 1), suggesting that lipofuscin accumulation is not related to OS phagocytosis, however, we
did not determine the A2E content of the fhRPE cells after termination of the experiment. I would hypothesize that the A2E content of the lipofuscin observed in the fhRPE not fed OS would be zero since A2E is derived from the precursor A2-PE found in the OS of photoreceptors until cleaved within RPE lysosomes to A2E (Ben-Shabat et al., 2002). There was no difference in lipofuscin formation between the OS fed fhRPE cells in the “dark” and “light” groups (Table 1, intraexperiment comparisons).

Table 1

Discussion

In this study, we have demonstrated that feeding fhRPE cells OS for 10-14 days in the absence of light increases lipofuscin formation by 1.4 to 4.5-fold, and melanolipofuscin formation by 2 to 2800-fold. In the absence of OS feeding, light alone increased the accumulation of lipofuscin by 3.6-fold (Experiment I) and 2.6-fold (Experiment II), with an accompanying increase in melanolipofuscin formation in Experiment I of 14.3-fold. In 1967, Young published that the rate of OS shedding increased in rats and frogs under conditions of continued high intensity illumination, and decreased in total darkness. Our data are consistent with this work, and are what we expected to see in the in vitro fhRPE cell culture system. The observed decrease in melanosome number with increased melanolipofuscin and lipofuscin granule numbers, except for the first 14-day experiment, is expected based on the known decrease in melanin number and increase in melanolipofuscin, melanolysosome, and lipofuscin pigments in the aging human RPE (Feeney-Burns et al., 1984). The lower number of
melanosomes in all fhRPE cells exposed to a 12-hour light/dark cycle, suggests that there is an increased use of the melanosome in generating the lipofuscin and melanolipofuscin granules – regardless of the presence of OS. However, more experiments must be performed in order to verify that melanosomes are used in the process of OS degradation. The experiments must be well planned and executed, and the volume of product (lipofuscin, melanolipofuscin, and melanosomes) is low – not allowing for easy purification or exhaustive characterization. These caveats are well noted but should not discourage the use of the fhRPE cell culture method from use in better understanding the role of the melanosome in the phagocytosis and degradation of OS. Our results support a role for the melanosome in degrading phagocytosed OS and in the production of the degradative intermediates melanolipofuscin and lipofuscin.
Fig. 1. Subcellular fractionation of fhRPE lysates on discontinuous sucrose gradients.
A. Density-gradient tube before centrifugation showing the layering of sucrose solutions and the membrane-free sample. The gradient was made immediately before loading the sample; and the gradient was centrifuged immediately after the sample was loaded.
B. Three fractions appeared in the gradient tube after ultracentrifugation at 103,000 X g for 2 hours. Images x400.

Fig. 2. Representative discontinuous sucrose gradient from a 57 year human donor eye, with accompanying fluorescence and DIC images of interface fractions. A. Transmitted light and fluorescence images of the sucrose gradient showing the lipofuscin, melanolipofuscin, and melanosomes. Only the lipofuscin fraction brightly fluoresces.
B. DIC and fluorescence images of harvested fractions. Lipofuscin intensely fluoresces.
Table 1  
Granule Counts (granules/mL)

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<th>Dark</th>
<th>+OS</th>
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<td>207 000 ± 55 302</td>
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<tr>
<td>Fraction #2 Melanolipofuscin</td>
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<td>0.05</td>
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<tr>
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<tr>
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<td>236 250</td>
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<td>72 000</td>
<td>16 625</td>
<td>56 100</td>
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<tr>
<td>Fraction #3 Melanosomes</td>
<td>2 720 000</td>
<td>1 128 750</td>
<td>1 150 000</td>
<td>1 207 500</td>
</tr>
</tbody>
</table>
Figure 1

**A**

- Membrane-free Sample
- 0.5 mL
- 1.0
- 0.5 mL
- 2.0
- 0.5 mL
- 2.5
- 0.5 mL

*Molarity of Sucrose Solution*

**B**

- #1 Lipofuscin
- #2 Melanolipofuscin
- #3 Melanosomes

*Interface Fractions*
Figure 2

A

Transmitted Light  Fluorescence

Lipofuscin

Melanolipofuscin

Melanosomes

B

DIC

Fluorescence

Fraction #1 (Lipofuscin)

Fraction #2 (Melanolipofuscin)

Fraction #3 (Melanosomes)
CHAPTER 4

PRESENT STUDY

Bestrophin is a Regulator of Cellular Ca++-Dependent Ion Conductance

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Abstract

Mutations in bestrophin-1, encoded by the BEST1 gene, cause three different retinal degenerative diseases including Best vitelliform macular dystrophy (BMD), an inherited maculopathy. BMD is differentiated from other maculopathies by a diminished electrooculogram (EOG) light peak (LP), which has given rise to the hypothesis that bestrophin-1 generates the LP. However, data from bestrophin-1 deficient mice have shown that bestrophin-1 is not required to generate the LP, though it may function to antagonize it. To investigate the basis of this, we examined the transepithelial electrical properties of primary fetal human RPE (fhRPE) cultures. These cultures express bestrophin-1 endogenously. Overexpression of bestrophin-1, or the mutants W93C or R218C, resulted in changes to the short circuit current (Isc) measured in Ringer’s solution, and changes in the response to ion substitution, and ionomycin and niflumic acid (NFA) stimuli. Despite these changes, the amount of bestrophin-1 in the plasma membrane was not altered by overexpression, and the polarity of bestrophin-1 did not differ from control cells. Based on these data I conclude that bestrophin-1 regulates Ca++-sensitive ion

...
transport, and that BMD is due to an innate activation of this function rather than to diminished bestrophin-1 Cl\(^-\) channel activity.
**Introduction**

Mutations in the gene *BEST1*, which encodes the protein bestrophin-1 (Petrukhin et al., 1998; Marquardt et al., 1998), cause three different retinal degenerative diseases: BMD, adult-onset vitelliform macular dystrophy (AVMD), or autosomal dominant vitreoretinalchoroidopathy (ADVIRC). BMD is distinguished from AVMD and other macular degenerative diseases by its only fully penetrant symptom; a LP to dark-trough ratio (the Arden ratio) of the EOG of less than 1.55, with a normal clinical electroretinogram (ERG)(Cross and Bard, 1974). Even asymptomatic carriers of BMD-associated mutations, assessed by pedigree, usually exhibit an altered EOG (Bard and Cross, 1975; Maloney et al., 1977). Bestrophin-1 is localized to the basolateral plasma membrane of the retinal pigment epithelium (RPE)(Bakall et al 2003; Chong et al., 2005; Marmorstein et al., 2000b). This is the site at which the EOG LP is generated by a depolarization of the basal plasma membrane due to activation of a Cl⁻ current (Gallemore and Steinberg, 1989a; Gallemore and Steinberg, 1989b; Gallemore and Steinberg, 1993; Gallemore et al., 1998). Based on these data it has been hypothesized that bestrophin-1 is a Cl⁻ channel and that it generates the LP. Evidence supporting the hypothesis that bestrophin-1 is a Ca⁺⁺-sensitive Cl⁻ channel has been provided by several laboratories and is based on heterologous expression of bestrophin-1 and other bestrophin family members. When expressed in HEK-293 cells, bestrophins appear to induce a Ca⁺⁺-activated Cl⁻ conductance that was not present in control cells. When BMD associated bestrophin-1 mutants were expressed in HEK-293 cells, the Cl⁻ currents were substantially smaller than in controls, and the mutants appeared to exert a dominant effect
when co-expressed with wild type bestrophin-1 (Chien et al., 2006; Fischmeister and Hartzell, 2004; Qu and Hartzell, 2004; Qu et al., 2003; Sun et al., 2002; Tsunenari et al., 2003; Tsunenari et al., 2006). The ion selectivity of mouse bestrophin-2 currents can be altered by selective mutagenesis (Qu and Hartzell, 2004; Qu et al., 2004; Qu et al., 2006), and the Ca\(^{++}\) sensitivity of the conductances has been demonstrated for bestrophin-1 using caged Ca\(^{++}\) (Sun et al., 2002) and for bestrophin-4 in excised patches (Tsunenari et al., 2006). Finally, single channel recordings associated with expression of *Drosophila* bestrophins have been reported, though these channels are ATP dependent, a property not previously associated with bestrophin currents (Chien et al., 2006). Despite this strong body of data, there is evidence that bestrophin-1 is not required to generate the LP (Marmorstein et al., 2006), and that bestrophin-1 is also a regulator of Ca\(^{++}\)-activated Cl\(^{-}\) channel (Cl\(_{Ca}\)) function (Marmorstein et al., 2006; Rosenthal et al., 2006). Data supporting this include the ability of bestrophin-1 to alter the kinetics of activation and inactivation of voltage-dependent Ca\(^{++}\) channels (Rosenthal et al., 2006), and the enhanced LP luminance response in bestrophin-1 deficient mice (Marmorstein et al., 2006).

To better understand how bestrophin-1 activity relates to disease pathogenesis, I sought to investigate the effects of overexpression of bestrophin-1 and the bestrophin-1 disease causing mutants W93C and R218C on the transepithelial electrical properties of fhRPE cultures. These cultures express the protein endogenously when grown on permeable supports that allow them to develop a tight epithelial monolayer. Overexpression of bestrophin-1, the W93C mutant, or the R218C mutant, did not change
cell surface expression or cellular polarity of the proteins compared to control cells expressing endogenous levels of bestrophin-1. However, overexpression of wild type bestrophin and bestrophin-1 mutants did alter the baseline transepithelial potential (TEP) and short circuit current ($I_{sc}$) of the monolayers, but not the transepithelial resistance (TER). I also observed that only fhRPE monolayers overexpressing bestrophin-1 exhibited a significant reduction in $I_{sc}$ when Cl$^-$ was substituted with D-gluconic acid. The changes in TEP and TER were the same across experimental groups. In addition to these findings, cells overexpressing bestrophin-1 showed a decrease in response to ionomycin (calcium ionophore) with a comparable NFA response compared to controls; while the mutants W93C and R218C showed significantly decreased ionomycin responses, and NFA responses were nearly abolished for W93C and R218C compared to controls. Based on these findings we conclude that bestrophin-1 regulates intracellular Ca$^{++}$-sensitive ion responses, and that the electrical parameters altered in BMD are due to an innate activation of bestrophin-1 associated Ca$^{++}$ regulatory activity rather than to a loss of bestrophin-1 Cl$^-$ channel activity.
**Experimental Procedures**

**fhRPE cell culture**

Fetal human RPE cultures were prepared and maintained according to the method of Hu and Bok, 2000. The tenets of the Declaration of Helsinki were followed, and parents gave consent for donation of tissue. For physiology experiments, cells were plated at a density of 200,000/cm² on 1cm² Millicell-HA culture inserts. TER was followed for each insert on a weekly basis using an epithelial voltageohmmeter (World Precision Instruments, New Haven, CT). All monolayers used were cultured for > 8 weeks unless otherwise indicated.

**Adenovirus constructs and transduction**

FhRPE monolayers with TERs > 500 Ω*cm² when measured in Ringers (see below) were transduced with a modified type 5 replication defective adenovirus vectors on the Ψ5 backbone. The adenovirus vectors for expression of best1, best1-W93C, and best1-R218C have been described previously (Marmorstein et al., 2004). For transduction, monolayers were rinsed with MEM (Cat.# M2279, Sigma, St. Louis, MO) and then 500 µL of vector diluted to an MOI of 3 was added to the apical chamber of the insert. After incubation at 37°C for 165 min, vector containing medium was removed and replaced with high Ca²⁺ maintenance medium (Hu and Bok, 2000) and the cells incubated for an additional 20-48 hrs prior to further use.

**Cell Surface Biotinylation, Immunoprecipitation (IP), and Western blots**

Domain selective biotinylation of the apical or basolateral plasma membranes of fhRPE monolayers on Millicell-HA inserts was performed as described previously.
Following biotinylation, cells were solubilized in Laemmli sample buffer, resolved by SDS-PAGE, transferred to PVDF, and blotted with antibodies against either bestrophin-1 (Mab E6-6)(Novus, Littleton, CO) or CD147 (BD Biosciences Pharmingen, San Diego, CA) alkaline phosphatase labeled secondary antibodies as described previously (Marmorstein et al., 2000b; Deora et al., 2004). Blots were developed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Promega, Madison, WI) at the volumes of 132 µL and 66 µL per 20 mL of alkaline phosphatase buffer (see manufacturer insert), and photographed on a Bio-Rad Chemidox Imaging station (Bio-Rad Laboratories, Inc., Hercules, CA). The intensity of bands was determined using Metamorph ver. 7.02 software (Molecular Devices Corp., Sunnyvale, CA).

To examine the level of bestrophin-1 expression following adenovirus transduction, monolayers were lysed and bestrophin-1 immunoprecipitated using a polyclonal antibody (Pab 125; Marmorstein et al., 2000). Immunoprecipitates were resolved by SDS-PAGE, transferred to PVDF, and probed with a monoclonal antibody (Mab E6-6)(Novus, Littleton, CO) against the C-terminus of human bestrophin-1 (Marmorstein et al., 2000b). Following incubation with alkaline phosphatase labeled secondary antibodies, the blots were developed as described earlier using NBT/BCIP (Promega, Madison, WI), digitized on a Bio-Rad Chemidox (Bio-Rad Laboratories, Inc., Hercules, CA), and band intensities determined using Metamorph ver. 7.02 software (Molecular Devices Corp., Sunnyvale, CA).
Electrophysiology

Experimental setup

All experiments were performed in a water jacketed Ussing chamber similar to that described by (Oakley, 1977; Miller and Steinberg, 1977) that was modified to hold Millicell HA cell culture inserts and containing larger bath volumes (8.5 mL apical, 8.0 mL basal). The bath temperature was maintained at 37°C by warming solutions with inline heaters and by use of a water jacketing system. Temperatures were monitored via thermistors placed in each bath. All experiments began by perfusing both sides of the chamber at a rate of 7 mL/min with Ringers solution containing in (mM): 113.4 NaCl, 5 KCl, 5.6 glucose, 0.8 MgCl₂, 2 glutathione, 1.8 CaCl₂, and 26.2 NaHCO₃, pH 7.4, until the TEP stabilized. All solutions were constantly gassed with 95% O₂, 5% CO₂. For the Cl-ion substitution experiments: monolayers were recorded in Ringers for 10 minutes, followed by perfusion with modified Ringers in which 90% of the normal Cl⁻ content was replaced by gluconate [in (mM): 108.9 D-gluconic acid (Na⁺ salt), 4.5 NaCl, 5 KCl, 5.6 glucose, 0.8 MgCl₂, 2 glutathione, 1.8 CaCl₂, and 26.2 NaHCO₃, pH 7.4] for 10 minutes, and then perfused again with Ringers for 10 minutes to determine the reversibility of the response. For experiments in which monolayers were stimulated with ionomycin and NFA: monolayers were maintained in Ringers for 10 minutes, after which the Ringer’s containing 500 nM ionomycin was perfused into the chamber. After 15 minutes, perfusion was continued using Ringer’s containing both 500 nM ionomycin and 100 µM NFA for an additional 10 minutes.
Recording configuration

Voltage across fhRPE monolayers (the TEP) was recorded using Ag/AgCl pellet electrodes bridged with agar 0.5 M KCl bridges in each bath. Signals were passed through a low pass Bessel filter, amplified using a DP-304 Differential Amplifier (Warner Instruments, Inc., Hamden, CT), digitized, and recorded and analyzed on a Dell PC using LabScribe 1.821 software (iWorx, Dover, NH). The TER of the monolayers was determined by passing bipolar 10 µA pulses (DS8000 Digital Stimulator, World Precision Instruments, Inc., Sarasota, FL) across the monolayer every 30 seconds between Ag-AgCl pellets – placed in each bath. TER was measured from the current-induced voltage changes across the monolayer. $I_{sc}$ was calculated as $I_{sc} = (TEP/TER)$, based on Ohm’s Law.
Results

Overexpression of bestrophin-1 and cellular polarity.

I used adenovirus mediated gene transfer to infect fhRPE monolayers cultured on permeable supports with bestrophin-1 or bestrophin-1 carrying the BMD associated mutations W93C or R218C. In all experiments, control monolayers were transduced with an equal MOI of a null virus (Ψ5) carrying no expression cassette. Transduction with adenovirus resulted in a 2-3 fold increase in bestrophin-1 expression compared to control monolayers using whole lysate preparations (Fig. 1A-B). To determine whether overexpression altered cell surface polarity of bestrophin-1, I performed domain selective biotinylation. Interestingly, bestrophin-1 was ~60% apical in all experimental groups. Densitometric analysis of total cell surface expression indicated that the amount of bestrophin-1 present in the plasma membrane was not increased due to overexpression (Fig. 1C-E).

Effects of bestrophin-1 and bestrophin-1 mutants on RPE transepithelial electrical properties.

The standing TEP and TER of fhRPE monolayers was determined as described in the methods and the Isc was calculated from those values. As shown in Figure 2A, control monolayers had a TEP of 2.23 ± 1.72 mV. Monolayers overexpressing bestrophin-1 had a significantly higher TEP than controls (P < 0.01). Those monolayers overexpressing bestrophin-1-W93C had an average TEP that was significantly lower than controls (P < 0.04) and those overexpressing bestrophin-1-R218C had TEPs that were the same as controls. On average, the TER of monolayers overexpressing bestrophin-1 or
bestrophin-1-W93C was similar to controls (Fig. 2B), and so differences in $I_{sc}$ paralleled those observed for TEP (Fig. 2C). For bestrophin-1-R218C, the TER was elevated compared to controls ($P < 0.02$). However, with $n = 7$ from two donors for bestrophin-1-R218C, I attribute this difference to the monolayers being from two donors having high TERs.

**Effect of Cl$^-$ substitution on transepithelial electrical properties.**

Since bestrophin-1 is a proposed Cl$^-$ channel, I hypothesized that $I_{sc}$ in monolayers overexpressing wild type (wt) bestrophin-1 would increase due to overexpression of an ion conducting channel. If more channels are present and conducting, then $I_{sc}$ should increase (see Fig. 5). I also hypothesized that the mutants W93C and R218C would exhibit inhibited Cl$^-$ transport, making them less sensitive to ion substitution. To test this, we replaced 90% of the total Cl$^-$ in our Ringers with gluconate. Figure 3A shows representative recordings from each experimental group. Despite an obvious and consistent increase in TER across all groups, a significant ($P < 0.05$) reduction in $I_{sc}$ was observed only for bestrophin-1 overexpressing cells (Wt), though bestrophin-1-W93C overexpressors also exhibited a non-significant reduced $I_{sc}$ (Fig. 3B). Return to Ringers solution resulted in a rapid reversal of the ion substitution effect for all experimental groups.
**Effect of ionomycin and NFA on transduced fhRPE monolayers.**

As bestrophin-1 is thought to be a ClCa, I examined the response of cells overexpressing bestrophin-1 and bestrophin-1 mutants to stimulation with the Ca++ ionophore ionomycin. Data from experiments examining the responses of control monolayers (Fig. 4A) revealed that increasing intracellular Ca++ by treatment with ionomycin resulted in a biphasic elevation of the TEP. The initial response, occurring between 2-3 minutes after stimulus onset, we call peak one (P1); and the secondary increase in TEP following P1, we call P2. The P2 response plateaued at 9-12 minutes after the application of ionomycin. Addition of 100 µM NFA, an inhibitor of ClCaS, resulted in a rapid reduction in TEP to a level near that of the P1 response. In contrast to the noticeable changes in TER elicited by Cl-ion replacement, no large sustained changes in TER were observed due to ionomycin or NFA. Overexpression of bestrophin-1 resulted in an increased initial TEP and Isc (Fig. 2A, 2C; Fig. 4A-C), but smaller P1 and P2 responses than observed for controls. Interestingly, the Isc following the P2 response was similar to that of control monolayers suggesting that ionomycin could not elicit a greater current despite bestrophin-1 overexpression, and the diminished Isc due to NFA was also similar to that of control monolayers, suggesting that bestrophin-1 overexpression functions more in a regulatory role for ion conductance than in the direct transport of Cl- ions (Fig. 4C). If bestrophin-1 was simply a Cl- channel, then overexpression of it should increase the Isc of responses involving bestrophin-1, or if the response were saturated, then the Isc observed should be at least the same at fhRPE expressing endogenous amounts of bestrophin-1 (refer to Fig. 5 of Chapter 1 for why an...
increase is $I_{sc}$ would be expected for overexpression of bestrophin-1). The results for mutant bestrophins were markedly different. Overexpression of bestrophin-1-W93C abolished P1 and P2, and reduced NFA responses (Fig. 4A). The increase in $I_{sc}$ at P2, and the decrease in $I_{sc}$ due to NFA in bestrophin-1-R218C overexpressing monolayers was similar in amplitude to that elicited in controls and in bestrophin-1 overexpressors (Fig. 4C). Though these changes in $I_{sc}$ at P2 and in NFA were similar, overexpression of bestrophin-1-R218C resulted in a significantly impaired P1 response, and a diminished P2 and NFA response (Fig. 4A). Figure 4B provides a schematic of how P1, P2, and the NFA response values were measured.
Discussion

Today, genetic testing is available that can provide an accurate diagnosis of BMD without the need for an EOG. However, in the past, the only fully penetrant symptom of BMD was a diminished LP of the EOG with a normal clinical ERG. The diminished LP of the EOG in BMD patients has often been cited as the basis for the hypothesis that bestrophin-1 is a Cl<sub>Ca</sub> and has led to a model in which BMD is due to impaired bestrophin-1 Cl<sub>Ca</sub> function (refer to The clinical ERG, the DC-ERG, and the EOG in the REVIEW OF LITERATURE section of Chapter 1)(Chien et al., 2006; Fischmeister and Hartzell, 2004; Qu and Hartzell, 2004; Qu et al., 2003; Sun et al., 2002; Tsunenari et al., 2003; Tsunenari et al., 2006). While this hypothesis is strongly supported by heterologous expression data, the recent finding that bestrophin-1 deficient mice have an enhanced LP luminance response (Marmorstein et al., 2006), and that bestrophin-1-W93C knock-in mice have an impaired luminance response function (our unpublished data) suggests that this hypothesis requires significant modification. Toward this end, I have chosen to study the effects of bestrophin-1 and mutant bestrophin-1 overexpression in fhRPE monlayers that express the protein endogenously. My data suggest that bestrophin-1 serves as a regulator of Ca<sup>++</sup> sensitive ion transport, and that an impaired ability to respond to Ca<sup>++</sup> stimuli underlies BMD.

As shown in Figure 1A and 1B, the transfection of RPE monolayers with adenovirus vectors directing the expression of bestrophin-1, bestrophin-1-W93C, or bestrophin-1-R218C, resulted in an increased overall level of bestrophin expression. Unexpectedly, bestrophin in these cells was ~60% apically polarized. This differs from
the basolateral polarization of bestrophin-1 that the Marmorstein lab and others have observed histologically in RPE cells in the eye (Bakall et al 2003; Mullins et al., 2005; Marmorstein et al., 2000b). Though this shift in bestrophin-1 polarization initially seemed problematic, it does not change how bestrophin-1 in the fhRPE monolayers will affect TEP. Recall that Cl⁻ transport across the RPE monolayer is observed as positive current that increases TEP when Cl⁻ transport of the monolayer is increased (Chapter 1 Fig. 5). Furthermore, since the 60% apical, 40% basolateral finding was consistent across all experimental groups – it will affect all the groups in the same way.

I was surprised to find that despite overexpression of the protein in whole cell lysates, the amount of bestrophin that was accessible to biotinylation was not increased, suggesting that the additional protein was not delivered to, or failed to be retained in the plasma membrane. Based on the ClCa hypothesis of bestrophin-1 function, this data led me to anticipate that there would be no difference between monolayers overexpressing bestrophin-1 and controls expressing endogenous levels of bestrophin-1. However, the Isc, in Ringers, of bestrophin-1 overexpressors was significantly greater than in all other experimental groups, indicating an increase in ion transport (Fig. 2C). That increase was likely due to Cl⁻, as substitution of gluconate for Cl⁻ resulted in a significant drop in Isc only in the bestrophin-1 overexpressors (Fig. 3B). However, this occurred without an increase in cell surface expression of the protein (Fig. 1C and 1E). With cell surface expression of bestrophin-1 and the bestrophin-1 mutants the same, the mechanism underlying how Cl⁻ transport is more greatly effected by transduction with wild-type
bestrophin-1 is not known, but this finding suggests that bestrophin-1 may be regulating Cl_{Ca} currents instead of being a Cl_{Ca}.

To further dissect the mechanism behind this increase in Cl⁻ transport, I examined the ability of the cell to respond to Ca^{++} by treating the cells with ionomycin, a Ca^{++} ionophore. I observed a biphasic increase in TEP without substantial change in TER, resulting in a biphasic increase in I_{sc} for control monolayers. Interestingly, the P1 was diminished in bestrophin-1 overexpressors. P2 was also diminished, but the maximum I_{sc} attained in the controls and overexpressors after ionomycin stimulation was similar (Fig. 4A and 4C). Treatment of these monolayers with NFA, an inhibitor of Cl_{Ca}S, diminished the I_{sc} of both controls and overexpressors equally. I interpret this data as indicating that bestrophin-1 regulates Ca^{++} sensitive transepithelial Cl⁻ transport. Since bestrophin-1 and bestrophin-1 mutants were shown to be overexpressed in whole lysate preparations, this result is most likely due to excess intracellular bestrophin-1 functioning to regulate Ca^{++}-sensitive ion conductances. Intracellular localization of bestrophins has been commonly observed in transduced HEK-293 cells (Qu et al., 2003; Stanton et al., 2006; Tsunenari et al., 2003), and is further supported by my finding that whole cell lysates show overexpression of the bestrophin-1 proteins, but that cell surface expression was not changed across experimental groups (Fig. 1).

Overexpression of bestrophin-1 mutants (W93C and R218C) that cause BMD, resulted in a significantly diminished response of the cells to ionomycin and NFA (Fig. 4A and 4C). Despite this, the I_{sc}, in Ringers, of bestrophin-1-R218C expressors was similar to controls, and the effect of Cl⁻ substitution with gluconate on these cells did not
differ from controls (Fig. 3A and 3B). For bestrophin-1-W93C, baseline $I_{sc}$ was diminished (Fig. 3B and Fig. 4C) and the ionomycin and NFA elicited responses were nearly abolished (Fig. 4C). I interpret this data as suggesting that bestrophin-1 mutants regulate Ca$^{++}$ homeostasis and Ca$^{++}$ stimulated ion transport and I hypothesize that bestrophin-1 is doing this by altering the RPE cell’s ability to use Ca$^{++}$ that is either entering the cells or that is released from intracellular stores such as the endoplasmic reticulum. Bestrophin-1 may be chelating Ca$^{++}$ itself by binding Ca$^{++}$ once it enters, or is released into, the cytoplasm.

But does this hypothesis explain data obtained in heterologous expression systems? Some aspects of my data are in agreement. For instance I do find that overexpression of bestrophin-1 results in an increased resting current ($I_{sc}$) due to enhanced Cl$^-$ transport. I also find that bestrophin mutants can suppress resting $I_{sc}$ (Fig. 2C, Fig. 3B, and Fig. 4C), as well as the ability of the cells to respond to Ca$^{++}$ stimuli (Fig. 4A and 4C). My data differs in that I have been able to test bestrophin-1 function in RPE cells that endogenously express bestrophin-1, whereas this is not possible in the HEK-293 cells (Fig. 3B). As my data show, overexpression of the bestrophin-1 mutants W93C and R218C did not significantly alter the response to Cl-ion substitution observed in controls with endogenous bestrophin-1 expression. It is possible that the increased Cl$^-$ currents observed in HEK-293 cells transduced with bestrophin-1 are due either to unmasking of an endogenous channel or to induction of gene expression, potentially via the effects of bestrophin-1 on Ca$^{++}$. In addition, my data suggest that if bestrophin-1 expression was diminished or abolished using RNA interference (RNAi) in
fhRPE, then an equal or diminished baseline $I_{sc}$ would be observed compared to control monolayers, and the cells would show an enhanced ability to respond to $Ca^{++}$ due to a decrease in the presence of bestrophin-1 to regulate $Ca^{++}$ within the RPE. Further study is required to test my hypothesis that bestrophin-1 regulates $Ca^{++}$ in the cytoplasm of the RPE.
Figure Legends

Figure 1. Bestrophin-1 protein expression and polarity in fhRPE. FhRPE infected with an empty cassette viral vector \( \Psi \)5 (Control), wild-type (Wt) bestrophin-1, bestrophin-1-W93C, or bestrophin-1-R218C. (A). Representative whole lysate immunoblots of bestrophin-1 and bestrophin-1 mutants in fhRPE. Cells were immunoprecipitated with Pab-125 antibody and blotted with E6-6 antibody. Each experiment was run in duplicate. Proteins visualized with AP-conjugated secondary antibodies. Bands shown are \( \sim \)68 kD. (B). Whole lysate quantification of bestrophin-1 and bestrophin-1 mutants. Blots from (A) were quantified using MetaMorph software, and the expression levels are presented versus Control. Wt, W93C, and R218C protein levels were all 2- to 3-fold higher than Control. (C). Representative immunoblots following biotinylation and immunoprecipitation with immobilized streptavidin beads. Paired samples were processed simultaneously, one following Apical (Ap) biotinylation, and the other following Basolateral (Ba) biotinylation – for each experimental group. Proteins visualized with AP-conjugated secondary antibodies to E6-6. Bands shown are \( \sim \)68 kD. (D). Quantification of apical versus basolateral expression of bestrophin-1 and bestrophin-1 mutants in fhRPE. Protein expression was consistent in all experimental groups (\( \sim \)60% Apical and \( \sim \)40% Basolateral). (E). Total cell surface protein expression presented as a percentage of Control expression. Total cell surface protein expression was not different between control and experimental groups. Bars in (B), (D), and (E) represent mean ± SD. (n = 4 for all groups)
Figure 2. Baseline characteristics of bestrophin-1 and bestrophin-1 mutants in fhRPE. FhRPE infected with an empty cassette viral vector Ψ5 (Control), wild-type (Wt) bestrophin-1, bestrophin-1-W93C, or bestrophin-1-R218C. (A) Transepithelial potential (TEP), (B) transepithelial resistance (TER), and (C) short circuit current (I_{sc}) of experimental groups. *P < 0.04 vs Control (A), *P < 0.02 vs Control (B) and (C). Bars represent mean ± SD. (n = 23 Control, n = 16 Wt, n = 11 W93C, n = 7 R218C)

Figure 3. Representative recordings (A) and short circuit current (I_{sc})(B) in response to replacement of Cl− with gluconate. FhRPE with only endogenous bestrophin-1 expression (fhRPE); or virally infected with an empty cassette viral vector Ψ5 (Control), wild-type (Wt) bestrophin-1, bestrophin-1-W93C, or bestrophin-1-R218C. (A). Verticle arrows indicate solution changes. Horizontal bars indicate where the average I_{sc} values were obtained for comparison between solution effects. The effects of replacement of Cl− with gluconate were reversible. (B). Bars indicate the I_{sc} recorded during the time indicated by the three horizontal bars in (A). *P < 0.03 vs Cl−. Bars represent mean ± SD. (n = 15 fhRPE, n = 20 Control, n = 14 Wt, n = 9 W93C, n = 4 R218C)

Figure 4. Representative recordings (A) and short circuit current (I_{sc})(B) in response to 500 nM ionomycin and 500 nM ionomycin + 100 µM niflumic acid (NFA). FhRPE infected with an empty cassette viral vector Ψ5 (Control), wild-type (Wt) bestrophin-1, bestrophin-1-W93C, or bestrophin-1-R218C. (A). Representative recordings showing the time of solution changes (long vertical arrows) and the locations of I_{sc} measurement
for Peak 1 (P1) and Peak 2 (P2) in response to ionomycin. Baseline $I_{sc}$ was averaged over the last 1½ minutes in normal Ringers (prior to addition of ionomycin); and for the effect of NFA, $I_{sc}$ was averaged over the last 1½ minutes of recording as in the traces shown in (A). (B). Schematic showing how P1, P2, and the NFA effect were measured. (C). Bars indicate the $I_{sc}$ recorded for each experimental group at baseline, P1, P2, and at the end of the recording for the effect of NFA. Differences between bars indicate the change in $I_{sc}$ between experimental conditions. *P < 0.05 vs Control (P1), *P < 0.02 vs Control (P2 and NFA). Bars represent mean ± SEM.
Figure 1.

A

Exp. 1
Exp. 2
Exp. 3

B

Level of Expression (% of Control)

C

Bestrophin

Ap  Ba  Control  Wt  W93C  R218C

D

(% Purity)

E

Cell Surface Expression (% of Control)
Figure 2.
Figure 3.
Figure 4.

A

+ Ionomycin (500 nM)
+ NFA (100 μM)

Control
Wt
W93C
R218C

5 mV
4 min

B

P1
P2

NFA Effect

C

I_{sc} (μA/cm²)

Control  Wt  W93C  R218C

Baseline  P1  P2  NFA
SUMMARY AND FUTURE DIRECTIONS

FhRPE cells have been used to study how best-1 and best-1 mutant overexpression alter Cl⁻ transport and Ca⁺⁺ responses in the RPE. This method of culture has also been used to develop a protocol for experiments investigating the formation and accumulation of lipofuscin within the RPE (APPENDIX C). Future experiments will continue to look at the role of the melanosome in this process, and how best-1 overexpression and best-1 mutant overexpression in fhRPE alter the rate of lipofuscin formation.

Overexpression of best-1 and the best-1 mutants W93C and R218C in fhRPE transduced with replication defective adenovirus vectors has been shown. The present study has also shown that the observed protein overexpression does not occur at the cell membrane, as all experimental groups had the same approximate 60% apical and 40% basolateral protein expression. This differs from the basolateral polarization of best-1 in vivo. Overexpression of best-1 resulted in noticeable changes in resting TEP and Iₛₑ. Noteable decreases in these two parameters were found in best-1-W93C overexpressors. Interestingly, best-1-R218C overexpressing fhRPE cells did not exhibit altered baseline electrical properties, complicating how to determine a common disease mechanism between W93C and R218C.

Cl⁻ ion substitution experiments showed that only best-1 overexpressors were significantly responsive to removal of Cl⁻ from the bathing Ringers, and this response
was reversible. The other experimental groups were not significantly responsive to Cl− substitution, or significantly different from one another in Isc operating within the cells.

To test the Ca++ sensitivity of best-1, best-1 and the mutants W93C and R218C were overexpressed in fhRPE and exposed to ionomycin to elicit Ca++-activated ion conductances. In the same experiments, the cells were treated with NFA following ionomycin to see how much of the Ca++-activated conductance was due to Cl− channels. It was found that control monolayers endogenously expressing best-1 had the greatest responses to Ca++ and NFA (see Chapter 4 PRESENT STUDY). Unexpectedly, best-1 overexpressors did not have responses that were greater than controls. These responses achieved the same maximal Isc values but P1 was almost completely diminished.

Interesting to me is that the baseline Isc for best-1 overexpressors started at the same Isc as the control monolayers achieved at P1. This data suggests a role for best-1 in inhibiting the RPE’s ability to respond to a Ca++ stimulus. This is supported further by the mutants, whereby R218C overexpressors didn’t have a P1 response, but had P2 and NFA responses similar to controls and wild type (wt), yet the amplitudes of the responses observed were diminished. W93C overexpressors were not responsive to Ca++ or NFA. The diminished responses of the R218C mutant, and especially the lack of responses to ionomycin and NFA by fhRPE expressing W93C, lead me to hypothesize that best-1 is regulating the RPE’s ability to utilize Ca++ once it has reached the cytoplasm – either by entering the cell or by release from intracellular stores. Best-1 may be chelating (binding) the Ca++ once it enters the cell. Further studies, listed later in this section, are required to test this hypothesis.
The data of the Present Study support previous studies on the function of best-1, but the interpretation cannot be taken as definitive. In relation to the Ca\(^{++}\)-sensitivity of Cl\(^{-}\) currents observed in transduced HEK-293 cells with the overexpression of best-1, my data may account for this by best-1’s ability to increase intracellular [Ca\(^{++}\)] ([Ca\(^{++}\)]\(_i\)) which could interact with an endogenous Cl\(^{-}\) channel of HEK-293 cells. Or the increase in [Ca\(^{++}\)]\(_i\) could be causing the transcription of a Cl\(_{Ca}\) that then induces the observed Cl\(^{-}\) currents. In RPE-J cells, overexpression of best-1-W93C resulted in a slowing of the activation and inactivation kinetics of VDCCs, and overexpression of best-1-R218C resulted in faster activation and inactivation VDCC kinetics (APPENDIX B). This data can be used to help explain the results of the present study and extend them to the rat model of BMD (Marmorstein et al., 2004). Endogenous best-1 expression in fhRPE maintains Ca\(^{++}\) homeostasis within the RPE, allowing the cells to respond to Ca\(^{++}\) stimuli. When overexpressed in RPE-J cells, best-1 was found to shift the voltage-dependence of VDCC currents closer to the resting potential of RPE (approx. -30 mV) and quickened the activation and inactivation kinetics of VDCCs (APPENDIX B). With cell surface expression being the same for controls, best-1, and best-1 mutants in the Present Study, an increase in the activation/inactivation kinetics of VDCCs would explain the same maximal response to Ca\(^{++}\) in best-1 overexpressors compared to controls, and would also support that more VDCCs would be open and conducting at rest. The same reasoning supports the data from Marmorstein et al., 2004, showing the same maximal LP responses achieved between control rats endogenously expressing best-1 and rats overexpressing best-1. It is gratifying to note that the I\(_{sc}\) presented in Figure 4C of the
Present Study mimicks the LP luminance responses reported in Figure 6A of Marmorstein et al., 2004 (see figure at the end of this section).

The activation kinetics of R218C overexpressed in RPE-J cells (APPENDIX B, Fig. 5) were faster than best-1 overexpressors and could account for similar Ca\textsuperscript{++} responses at P2 and NFA compared to best-1 overexpressors in the Present Study. The increased activation/inactivation kinetics could be fast enough that the I\textsubscript{sc} of the RPE at rest is not different than controls. In addition, R218C expressors completely inactivate, whereas bestrophin-1 overexpressors inactivate to approximately 13% of their maximal response, and W93C expressors inactivate to approximately 40% of their maximal response (APPENDIX B, Fig. 5A-C). By similar reasoning, the kinetics of the VDCCs caused by the mutant W93C, as reported in APPENDIX B, could be slow enough so that only a certain percentage of VDCCs can be active and conducting at any time and agonized by an inactivating background Ca\textsuperscript{++} current in these cells (APPENDIX B, Fig. 5B).

I must also note here that for rapid changes in intracellular Ca\textsuperscript{++} homeostasis to occur in the RPE, L-type VDCCs are antagonized by outwardly rectifying K\textsuperscript{+} channels (Hughes and Steinberg, 1990; Hughes et al., 1995; Strauss et al., 1993; Strauss et al., 2002; Takahira and Hughes, 1997; Tao et al., 1994; Wen et al., 1993). These outwardly rectifying K\textsuperscript{+} channels activate more slowly than the L-type VDCCs. Therefore, an influx of Ca\textsuperscript{++} through VDCCs can occur and depolarize the RPE cells until it is terminated by activation of these K\textsuperscript{+} channels, leading to hyperpolarization of the RPE. This regulation by the outwardly rectifying K\textsuperscript{+} channels prevents a toxic overload of Ca\textsuperscript{++}
in the cell after VDCC activation. This antagonism further supports the behavior of the
bestrophin-1 mutants. R218C expressors most likely inactivate before outwardly
rectifying K⁺ channels activate, and W93C expressors, with their constant non-
inactivating Ca⁺⁺ current cannot respond to Ca⁺⁺ influxes because recruiting additional
VDCCs is not possible. The comparison of APPENDIX B, Marmorstein et al., 2004,
Marmorstein et al., 2006, and the present study provide the strongest support for best-1
functioning to regulate Ca⁺⁺ homeostasis within the RPE, and indirectly affecting Cl⁻
conductance across the monolayer via ClCa channels; bringing the Cl⁻ conductance across
the basolateral membrane in full circle with it generating the LP. And if best-1 is not
present, as in the best-1 KO mouse, the LP is maximal in response and seemingly
uninhibited.

So how does a diminished P1 response for best-1 overexpressors and the mutants
fit into the picture? Is P1 required to generate P2? The answers are not known. One
study using 50-100 µM ATP (or UTP) to stimulate the release of Ca⁺⁺ from the
endoplasmic reticulum also produced large, biphasic voltage responses similar to the TEP
data of the Present Study (Peterson et al., 1997). This data was obtained from
intracellular recordings on freshly dissected bovine RPE sheets. The observed biphasic
voltage responses could be attenuated with the Ca⁺⁺ chelator BAPTA, or by treatment
with the non-specific anion channel blocker 4,4’-diisothiocyanostilbene-2,2’-disulfonate
(DIDS). Treatment with 3 mM DIDS at the basolateral membrane produced a 75%
reduction in the biphasic voltage responses, indicating that both phases of the response
were attributable to an increase in basolateral membrane Cl⁻ conductance (see The
clinical ERG, the DC-ERG, and the EOG in the REVIEW OF LITERATURE).

Additional experiments were also able to reduce the ATP-induced responses by treatment with the ATP-receptor subtype (P₂-purinoceptor) blocker suramin. In addition, another study using a human RPE derived cell line (ARPE-19) and monitoring [Ca⁺⁺], using fura-2, were able to stimulate a biphasic release of ATP from the apical membrane of the cells that corresponded to a biphasic rise in [Ca⁺⁺], by treatment with 100 µM to 1 mM uridine triphosphate (UTP)(Mitchell, 2001). The second phase of this response was inhibitable by treatment with 100 µM 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), a Cl⁻ channel blocker. The experiments performed measured Ca⁺⁺ responses directly and not the voltage responses of the cells, so how these findings relate to that of Peterson et al., 1997, and the Present Study, I cannot determine (outside of the observed Ca⁺⁺ stimulated responses).

With further thought, I propose that P₁ in my experiments is created by Ca⁺⁺ entry into the fhRPE cells following exposure to ionomycin and that P₂ is created by an additional effect of best-1 on ion transport across the RPE monolayer, most likely ClCa conductance (based on the work presented in the Present Study and in the REVIEW OF LITERATURE). This is supported by research showing that ionophore-induced resistance decreases and voltage increases should be monophasic and reach steady-state values. Secondary changes in resistance or voltage suggest changes in the resistors of the apical and/or basolateral plasma membranes produced by primary or secondary effects of the ionophore (Lewis and Wills, 1989).
I hypothesize that disruption of Ca\(^{++}\) homeostasis in the RPE could be the link between the EOG abnormality in BMD patients and the increased accumulation of lipofuscin in patients harboring specific BMD causing mutations. A decrease in the ability of RPE cells to respond to Ca\(^{++}\), or to increased [Ca\(^{++}\)]\(_{i}\), may result in a decreased ability of the RPE to phagocytose OS; or could decrease the ability of lysosomes, melanosomes, and phagosomes to interact and for vesicles carrying degraded material to fuse with the basolateral membrane for secretion into the bloodstream (leading to lipofuscin accumulation within the RPE).

The author feels that future research should follow a five-pronged approach:

1) Studies should be done to more accurately determine the structure of best-1

2) RNAi could be used to knock-down endogenous expression of best-1 in fhRPE. This is a critical next step to see if knocking-down best-1 expression results in fhRPE having even greater responses to Ca\(^{++}\) and NFA. (This would support the best-1 KO mouse model LP luminance response data.)

3) Additional best-1 mutants could be studied using the fhRPE cell culture method [Ex.: A243V – known to cause AVMD with no EOG defect; or mutants Q293K, G299E, E300D, D301E, and T307I – mutants in the putative fourth transmembrane domain (TMD4) and proximal carboxy tail that may behave in whole monolayers of fhRPE much differently than W93C (close to TMD2 on the intracellular side)]
and R218C (between TMD2 and TMD3) because they are located near TMD4 at the beginning of the cytoplasmic carboxy tail, they haven’t been tested in fhRPE, and any change in the amplitude of responses observed in experiments (as long as there are response) could provide information regarding disease severity in patients with these mutations.]

4) Additional experiments need to be performed looking at how Ca$$^{++}$$ behaves within the RPE

a. Is resting [Ca$$^{++}$$]i different in fhRPE endogenously expressing best-1, overexpressing best-1, or overexpressing best-1 mutants?

b. Do the Ca$$^{++}$$ responses differ between Ca$$^{++}$$ influx from outside the cells versus stored-release of Ca$$^{++}$$ from within the cells?

The work of Peterson et al., 1997, and this dissertation work show similar results, but knowing how the results differ within the same cell culture method would be informative – especially using fhRPE cells. This work would have to include stimulation with ATP, followed by experiments pretreating with ATP-receptor agonists and/or antagonists, or endoplasmic reticulum Ca$$^{++}$$-ATPase agonists and/or antagonists. These studies would be most insightful if performed using
intracellular recordings due to the complexity of interpreting the data (Peterson et al., 1997).

c. Are Ca\textsuperscript{++} binding proteins like calmodulin involved in the mechanism of best-1 modulation of VDCCs? Is the calmodulin-dependent protein kinase (CaMKII) involved?

5) Further studies should be performed using the methodology presented in Chapter 3, investigating how best-1 overexpression, and overexpression of best-1 mutants, affects lipofuscin accumulation within fhRPE and how the melanosome may or may not be involved in the process.
REFERENCES


APPENDIX A:

FOCUS ON MOLECULES: BESTROPHIN (BEST-1)

Key Words: Electrooculography, Electroretinography, Lipofuscin, Macular Degeneration, Retinal Pigment Epithelium.

Accession numbers: (complete cds) NM_004183; AF073501; BC015220; BC041664

Structure: Encoded by the VMD2 gene on chromosome 11q13 Best-1 is the prototypic member of the RFP family of proteins which are more commonly called “bestrophins”. The protein family was originally identified in C. elegans based on a conserved amino acid (aa) motif Arg-Phe-Pro (RFP). In humans there are 4 members of the bestrophin family numbered sequentially Best-1 through Best-4. Bestrophins all contain a conserved domain of ~310 aa which begins at their respective N-termini and contains 4 putative transmembrane helices (TM, figure 1). In Best-1, the extracellular loops between TM-1 and TM-2, and TM-3 and TM-4, are 20 and 17 amino acids respectively. A large cytoplasmic loop of ~140 aa separates TM-2 and TM-3. A highly variable cytosolic domain which follows TM-4 distinguishes the members of the family from each other. Best-1 reportedly forms homo-oligomers, however the stoichiometry of these oligomers has not been fully resolved.
**Function:** Best-1 has a very limited tissue distribution with mRNA having been identified only in the retinal pigment epithelium (RPE), testis, placenta, and brain, and protein having been detected only in the RPE where it is localized to the basolateral plasma membrane. The light peak (LP) of the electrooculogram (EOG) is generated by a Cl⁻ conductance across the basolateral plasma membrane of the RPE. Since LP defects are a characteristic of Best Vitelliform Macular Dystrophy (BMD), a disease caused by mutations in Best-1, it was hypothesized that Best-1 functions as a Ca²⁺ sensitive Cl⁻ channel (CaCC) that generates the LP. Whole cell patch clamp studies of Best-1 and other bestrophins heterologously expressed in cultured cells support this hypothesis (Sun et al. 2002). Further support comes from experiments in which replacement of key amino acids appears to alter the channel ion selectivity [reviewed in (Hartzell et al., 2005)]. The LP, however, exhibits increased luminance sensitivity in Vmd2 knock-out mice and alterations in the Ca²⁺ response evoked by ATP without any obvious effects on Cl⁻ conductances (Marmorstein et al., 2006). Furthermore, the LP is desensitized when Best-1 is overexpressed in rats. Thus, Best-1 appears as an antagonist of the EOG light peak, not the generator. Recently, Rosenthal et al., (Rosenthal et al. 2006) found that Best-1 can modify the kinetics of voltage dependent Ca²⁺ channels (VDCCs). Interestingly, the BMD associated mutations W93C and R218C altered VDCC kinetics different from each other and wild-type Best-1. The relationship between Best-1’s function as a CaCC and its ability to alter VDCC kinetics and Ca²⁺ signaling requires further study.
Disease Involvement: Mutations in the \( VMD2 \) gene resulting in changes to the primary structure of Best-1 have been identified in 3 diseases; BMD, (http://www3.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=153700), adult-onset vitelliform dystrophy (AVMD, http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=608161), and autosomal dominant vitreoretinalchoroidopathy (ADVIRC, http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=193220). All of the above diseases exhibit a dominant pattern of inheritance. No disease caused by \( VMD2 \) with a recessive pattern of inheritance has been identified to date, and studies of \( Vmd2 \) deficient mice indicate that the absence of Best-1 is well tolerated (Marmorstein et al., 2006). At least 95 different mutations causing BMD and/or AVMD have been described. These are summarized at the \( VMD2 \) mutation database (http://www.uni-wuerzburg.de/humangenetics/vmd2.html). Of these mutations (figure), 92 are single aa substitutions or deletions occurring at one of 68 different positions in the conserved RFP-domain of the protein. One is at a splice site and two are frame shifts. In ADVIRC, 3 mutations resulting in aa substitutions and possibly exon skipping have been described. All three amino acids are in TM domains. Mutations in these 3 aa have not been attributed to BMD or AVMD. With only 2 exceptions all of the mutations causing BMD, AVMD, and ADVIRC are found in four clusters occurring in the cytoplasmic region of the protein near each TM helix, or within the TM helix itself (see figure).

Clinically, BMD and AVMD are characterized by vitelliform lesions in the ocular fundus. At early stages, the yellow lesion has an appearance similar to that of an egg-yolk, which,
as the disease advances becomes “scrambled”. In BMD this lesion may occur as early as the first decade while in AVMD it is undetected until the fourth or fifth decade. BMD and AVMD are distinguished clinically by electrophysiological testing. The electroretinogram (ERG) of patients with both BMD and AVMD is typically normal, however, the ratio of the LP to dark trough of the EOG is markedly diminished in BMD. The histopathologic consequences of BMD and AVMD are similar and include accumulation of lipofuscin, RPE hypertrophy, sub-retinal and occasional sub-RPE deposits.

The fundus appearance of ADVIRC includes an abnormal zone of hyper and hypo-pigmentation between the equator. Cystoid macular edema is often observed. While EOG abnormalities have been reported in ADVIRC they are typically accompanied by ERG abnormalities as well. The histopathology of ADVIRC includes RPE cells of markedly irregular thickness and pigmentation, accumulation of lipofuscin in RPE cells, loss of photoreceptor outer segments, RPE atrophy, and proliferation of glial cells resulting in preretinal membranes.

**Focus of Future Studies:** Understanding the function of Best-1 and how mutations in the protein cause disease is essential to developing treatment strategies for BMD, AVMD, and ADVIRC. While data supporting the hypothesis that Best-1 is a CaCC are compelling, two mutations have been shown to cause both AVMD and BMD. Furthermore from our studies of *Vmd2* knock-out mice, we conclude that Best-1 is not the LP generator, but rather functions as a modifier of the light peak luminance response.
possibly via its ability to alter RPE Ca\textsuperscript{++} responses. Thus, further studies are necessary to
determine whether Best-1 forms a channel pore, and/or as data regarding VDCCs and the
\textit{Vmd2} deficient mouse would suggest, is a regulatory component of Ca\textsuperscript{++} signaling. Along
this line, it is important to understand the mechanism by which Best-1 alters the kinetics
of VDCCs and intracellular Ca\textsuperscript{++} responses. Reconciling any relationship between CaCC
activity and Ca\textsuperscript{++} modulatory activity should have a high priority. While every member of
the bestrophin family tested to date has been associated with \textit{de novo} CaCC activity, and
all exhibit unique I/V relationships, the sensitivity of bestrophins to CaCC specific
inhibitors (ie. niflumic acid) and the single channel characteristics have not yet been
reported. A comprehensive description of the pharmacology of putative bestrophin
channels, single channel recordings, and experiments using planar lipid bilayers would
seem essential. Perhaps the greatest challenge will be to understand the relationship
between Best-1 dysfunction and the histopathological consequences of diseases
associated with the \textit{VMD2} gene. Based on the available data, the relationship between
Best-1 dysfunction and the accumulation of lipofuscin and/or pigment defects in the RPE,
as well as the interaction of Best-1 with the LP are still open questions that must be
addressed.

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We apologize to the authors of the many important studies that are referred to but which we could not cite due to space limitations imposed by the journal.


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99: 4008-4013
**Figure Legend:** Putative structure of human Best-1. The protein is predicted to form 4 transmembrane helicies with both the N and C-termini within the cytoplasm. Individual mutations associated with BMD, AVMD, or ADVIRC are indicated.
APPENDIX B:

EXPRESSION OF BESTROPHIN-1, THE PRODUCT OF THE VMD2 GENE, MODULATES VOLTAGE-DEPENDENT CA\textsuperscript{2+} CHANNELS IN RETINAL PIGMENT EPITHELIAL CELLS

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Abstract

Mutations in the VMD2 gene cause Best’s disease, an inherited form of macular degeneration. The reduction in the light-peak amplitude in the patient’s electro-oculogram suggests that bestrophin-1 influences the membrane conductance of the retinal pigment epithelium (RPE). Systemic application of the L-type Ca\textsuperscript{2+} channel blocker nimodipine reduced the light-peak amplitude in the rat electroretinogram but not a- and b-waves. Expression of bestrophin-1 in a RPE cell line (RPE-J) led to changes in L-type channel properties. Wild-type bestrophin-1 induced an acceleration of activation kinetics of Ba\textsuperscript{2+} currents through L-type Ca\textsuperscript{2+} channels and a shift of the voltage-dependent activation to more negative values, closer to the resting potential of RPE cells. Expression of bestrophin-1 with Best disease causing mutations led to comparable shifts in voltage-dependent activation but different effects on activation and inactivation kinetics. Bestrophin W93C exhibited slowed activation and inactivation, and bestrophin R218C accelerated the activation and inactivation. Thus, transfection of RPE cells with bestrophin-1 distinctively changed L-type Ca\textsuperscript{2+} channel kinetics and voltage-dependence. Based on these data, we propose that presence of bestrophin-1 influences kinetics and voltage-dependence of voltage-dependent Ca\textsuperscript{2+} channels and that these effects might open new ways to understand the mechanisms leading to retinal degeneration in Best’s disease.

Key words: L-type Ca\textsuperscript{2+} channel, RPE, heterologeous expression, mutant bestrophin-1 R218C and W93C, light-peak
Introduction

Best’s vitelliform macular dystrophy is an autosomal dominant inherited form of macular degeneration with similarities to age-related macular degeneration (1-3). The disease is characterised by an early onset and a reduction in the light-peak in patients electro-oculogram (EOG) (1).

Best’s disease is caused by mutations in the VMD2 gene (4-12). The gene product of VMD2, bestrophin-1, is known to be exclusively expressed in the RPE (13). There, bestrophin-1 is localised to the basolateral plasma membrane (13) and interacts physically with protein phosphatase 2A (14). Bestrophin-1 belongs to the family of RFP-domain (an invariant arginine-phenylalanine-proline tripeptide) proteins (11, 15). Four members of this family are known (11, 16). Expression studies in HEK 293 cells indicated that bestrophin-1 and other RFP family members function as Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels (17-21). To date, all disease causing mutations in bestrophin-1 reportedly have led to a loss of Cl\textsuperscript{-} channel function with a dominant negative effect (17). These observations are proposed to explain the reduction in the EOG light-peak that is diagnostic for Best’s disease.

The light-peak is thought to arise from a substance designated “light-peak substance” which is released from inner layers of the retina in response to a light stimulus (22-25). The identity of the light peak substance is unknown. However, a leading candidate is ATP. The light-peak substance diffuses to the RPE where it presumably activates a signal transduction cascade. Based on studies of ATP stimulation, this most likely involves the inositol-1,4,5-trisphosphate/Ca\textsuperscript{2+} second-messenger system. In consequence, the Cl\textsuperscript{-} conductance across the basolateral plasma membrane is increased (22). Due to the high intracellular Cl\textsuperscript{-} activity in between 40-60 mM (22, 26, 27) the equilibrium potential for Cl\textsuperscript{-} is approximately in the range
between –30 to –20 mV which is more positive than the reported resting potential between
–45 to –40 mV in RPE cells (26, 27). The increased basolateral Cl- conductance causes a
depolarisation of the basolateral membrane which can be recorded as the light-peak (24).
Although the Cl- conductance which generates the light peak has been proposed to be Ca2+
sensitive, that hypothesis has never been experimentally tested. We have shown that RPE
cells express L-type Ca2+ channels, and that these channels provide a route by which
extracellular Ca2+ enter the cell and lead to the activation of Cl- channels in response to an
increase in intracellular inositol-1,4,5-trisphosphate (28).
Based on the hypothesis that bestrophin-1 represents the Ca2+-dependent Cl- channel activated
by the light-peak substance, it has been proposed that the loss of Cl channel function in the
mutant forms of bestrophin-1 directly leads to the primary symptom of Best’s disease, a
diminished light peak. However, characterization of patients with new VMD2 mutations
indicates that bestrophin-1 might not only function as a Cl- channel (29-34). These studies
described patients with late onset vitelliform macular degeneration, patients which show
onset of light-peak reduction after the onset of clinically identifiable macular degeneration or
patients with normal light-peak. These observations have been made with patients carrying
mutations which have been already published or so far unknown mutations. Furthermore, we
have recently shown that overexpression of bestrophin-1 in the rat did not increase maximum
light-peak amplitude but rather altered the kinetics of light-peak activation and the light
sensitivity of the response (35).
In order to identify other ion channels involved in the generation of the light peak we
performed light-peak measurement in the rat DC-ERG. To correlate these findings with
additional functions of bestrophin-1 we transiently transfected RPE-J cells, a rat RPE derived
cell line, with bestrophin-1. Except for primary RPE cultures established by the method of Hu and Bok (36), cultured RPE cell lines do not express a detectable level of endogenous bestrophin-1. Therefore, RPE cells lines represent an ideal tool to study bestrophin-1 function in RPE cells by transient transfection experiments (13, 36). Our findings suggest that bestrophin-1 alters several aspects of L-type Ca\textsubscript{2+} channel activity in RPE cells.
Material and Methods

Conventional ERG recordings
Rat DC-ERGs were recorded from both eyes simultaneously in response to strobe flashes ranging from –3.6 to 2.1 log cd sec/m² using a standard protocol (37). For ERG recordings animals were kept under anaesthesia using ketamine/xylazine (50mg/kg and 2mg/kg) as previously described (38). Rat experiments were performed in accordance to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The amplitude of the a- and b-waves was measured conventionally and the b-wave intensity-response function was analyzed using the Naka-Rushton equation (39). Recordings of the light-peak (LP) were made as described previously (38). The LP was recorded in response to a 5-min stimulus. The recorded signals were: c-wave followed by a negative fast oscillation and subsequent slowly developing signal in positive direction, the LP. From the baseline the LP appeared as signal in the negative voltage-range. LP amplitudes were determined by analysis of the absolute minimum and maximum values. Grand averages were derived by averaging all waveforms within each experimental group.

Cell culture and transfection
The rat RPE cell line RPE-J was purchased from ATCC. Cell culture and transfection. RPE-J cells were cultured in Dulbecco’s Minimal Essential Medium (DMEM supplemented with 4% fetal calf serum). The cultures were maintained at 32° C and 5 % CO₂ in air, and the medium was changed twice a week. Confluent cells were split in the ratio 1:2 using the trypsin/EGTA method. Subconfluent cultures were transfected using Lipofectamine
(Invitrogen) according to manufacturer’s instructions. The total amount of DNA per well (~1.5 cm) was 0.4 µg. Cotransfection of different bestrophins with eGFP was performed in a ratio of 4:1. Successful transfection of cells was monitored by fluorescence microscopy. Transfection efficiency ranged from 60-80 %.

Successful transfection of cells was monitored by fluorescence microscopy. Transfection efficiency was the same with either WT bestrophin-1, eGFP, W93C or R218C. Transfected cells were investigated between 24 and 72 h after transfection. During this time, no decrease in transfection rate could be observed.

**Plasmid Constructs**

All different bestrophins were cloned into pCDNA3.1. The plasmid pCDNA3.1-Best and the cloning method were previously described (13). The W93C and R218C mutants of bestrophin-1 were produced using the QuickChange site directed mutagenesis method (Stratagene).

**Patch-Clamp recordings**

Patch clamp recordings were made in the perforated patch configuration as described previously (40, 41). To isolate currents through L-type channels the following K+-free solutions were used (concentrations are in mM), bath solution: 130 NaCl, 3 TEACl, 10 BaCl2, 0.3 CaCl2, 0.6 MgCl2, 14 NaHCO3, 1 Na2HPO4, 33 HEPES, 6 glucose (pH = 7.2 with Tris); pipette solution: 100 CsCl, 10 NaCl, 0.5 CaCl2, 2 MgCl2, 5.5 EGTA, 10 HEPES (pH = 7.2 with Tris) and 150 µg/ml nystatin. The bath solution contains the salts of the cell culture medium (HEPES was used for iso-osmolar replacement of FCS and nutrients) to avoid volume regulatory responses by the cells after transfer from the cell culture medium to
the patch-clamp bath solution. To keep the activity of Ca$_{2+}$-dependent Cl- channels to a minimum the intracellular Ca$_{2+}$-concentration was adjusted to 10 nM. Membrane capacitance and access resistance were compensated for by the patch-clamp amplifier. In a minority of cells membrane capacitance could not be fully compensated for due to space clamp artefacts. These cells were excluded from kinetic analysis. To activate currents through L-type channels the cells were depolarised from a holding potential of –70 mV. Depolarisation consisted of 9 voltage-steps of 50 ms duration and 10mV increasing amplitude. Kinetic analysis of current data was as performed as indicated in Table 1. Voltage-dependent activation was analysed by plotting current amplitudes normalized to maximal current amplitude versus test potential. The resulting curve was fitted using the Boltzmann equation and corresponding fits were made for each cell. To compare the voltage-dependent activation between the cells, we used the potentials of the half maximal activation which were calculated from the Boltzmann fits. Activation kinetics, inactivation time constants and maximal current amplitudes were estimated from currents activated by a voltage-step from –70 mV to +10 mV. As an easy and reliable method, activation kinetics were evaluated by measurements of the time to peak of the current at ± 10 mV. In order to compare the activation kinetics with published data from other groups, some of the currents were analysed by calculation of the activation time constant according to the method used by Koschak et al. (42).

**Western Blot Analysis**

Western Blot analysis were performed as previously described in detail (43). Membrane lysates of confluent RPE-J cells were established. To the lysis buffer (20 mM Tris, 5 mM
MgCl₂, 1 mM EDTA, 0.3 mM EGTA) protease inhibitors (16 μg/ml benzamidine-HCl, 10 μg/ml phenanthroline, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 174 μg/ml phenylmethylsulfonylfouride, 1 μg/ml aprotinin) were added to avoid degrading processes. After three freezing and thawing steps (liquid N₂; 42°C) and two centrifugation steps at 500 and 43,000 g, the pellet was suspended in lysis buffer and subjected to SDS-PAGE (8.5% gel) and the proteins were blotted to nitrocellulose filter membranes (Polyscreen; NEN Life Science Products, Boston, MA). After blocking in 5% non-fat dry milk and 5% bovine serum albumin and incubation with primary antibodies, blots were finally visualized with a peroxidase-conjugated secondary antibody and a chemiluminiscence kit according to manufacturer’s instructions (Lumi light western blotting substrate, Roche). The images were digitalized using an image analyzer (Fujifilm; LAS 1000; Fuji, Tokyo; Japan) and suitable software (Aida 2.1; Raytest, Berlin, Germany). Anti-CaV1.2 and anti-CaV1.3 antibodies were purchased from Alomone Laboratories (Jerusalem, Israel).

RT PCR

Total RNA was extracted from RPE cells using the RNeasy Mini Kit (Qiagen) according to the manufacturers protocol. The RNA (1 μg) was reverse transcribed at 37°C for 1 h in the following reaction mixture: 1 μg oligo dT primer (Invitrogen), 1 mM of each dNTP, 20 U RNAguard (Amersham Biosciences), 20 U M-MLV reverse transcriptase (Invitrogen). For control PCR reactions human total brain RNA (Stratagene) was reverse transcribed under the same conditions.

PCR amplification was performed in a reaction mix containing 0.5 μl cDNA, 15 pmol of each amplification primer, 0.2 mM of each dNTP and 5 U Taq DNA polymerase. 40 cycles with
three temperature steps were used for amplification (94°C, 62°C, 72°C for the exons 11 and 44, and 94°C, 60°C, 72°C for the exon 32). The primers used were for exon 11 forward 5’-acgaggagattccagaagctc-3’ and reverse 5’-ggtgtgattgtagtgctcagagga-3’, for exon 32 forward 5’-gatgccatggacattctgaacatg-3’ and reverse 5’-gaggaggccacatacgggag-3’ and for exon 44 forward 5’-gcattgggaacctcagacagtgtcgtcctg-3’ and reverse 5’-gcggagctgctatcctcgtage-3’. Amplified DNA fragments were analysed by agarose gel electrophoresis and sequenced.

**Protein localisation by biotinylisation**

RPE-J cells were co-transfected to express GFP and one of the following: empty vector, bestrophin-1, bestrophin-W93C, or bestrophin R218C. After 24hrs, cells were surface biotinylated using sulfo-NHS-LC-biotin as described previously(44). Following biotinylation, bestrophin-1 or GFP were immunoprecipitated and the immunoprecipitates blotted as described previously (13) with streptavidin conjugated to alkaline phosphatase or monoclonal antibodies recognizing bestrophin 1 (E6-6) or GFP (Clontech) followed by alkaline phosphatase conjugated goat anti-mouse IgG. Blots were developed using TNBT and BCIP and photographed using a Biorad Chemidoc imaging workstation.

**Calculations and statistical analysis**

Data are presented as mean ± SEM and were analyzed for significance using ANOVA. Significance was assumed at the P-values of *P<0.05, **P<0.01, and ***P<0.001 with Sigma-plot software (Sigma Plot Scientific Graph System, Version 1.02, Jandel, San Rafael, Ca, USA). Mean values of data from Boltzmann fits were calculated from individual fits for
each experiment. All Western blot experiments were performed 3-4 times. Figure 2 shows one representative experiment.
Results

Presence of L-type channels in the RPE: effects of nimodipine on the light-peak

In order to determine whether RPE L-type Ca\text{2+} channels participate in generating the light peak, we analysed the effects of nimodipine, a specific inhibitor of L-type voltage gated Ca\text{2+} channels, on RPE-derived ERG components recorded from rats (Figure 1). Nimodipine is known to inhibit L-type channels without having systemic cardio vascular side effects.

Rats were injected with nimodipine (1mg/kg or 2.4 μmol/kg body weight) and dark adapted for 30 min after which ERGs were recorded in response to a 5 min 2.7 log cd m\text{2} stimulus. As shown in Figure 1B, RPE generated ERG components were smaller in nimodipine-treated animals than in vehicle-treated controls. In comparison, a- and b-waves were unaffected in rats receiving nimodipine (Fig. 1A, C, D) indicating that the reduction in RPE generated components induced by nimodipine was not due to suppression of phototransduction. An effect on the c-wave was not observed. On average, LPs in animals treated with nimodipine \((n=8 \text{ in each group})\) were reduced by \(-35\%\) \((p < 0.01)\) compared to animals treated with vehicle alone (Figure 1E), indicating a significant role for L-type Ca \text{2+} channels in determining LP amplitude.

RPE-J cells express L-type channels

To dissect the potential for bestrophin-1 to affect L-type Ca\text{2+} conductance we choose to examine RPE-J cells. First we determined whether RPE-J cells express L-type channels using whole cell patch clamp experiments. In the presence of 10 mM Ba\text{2+} in the bath solution RPEJ cells responded to depolarisation from a holding potential of \(-70\) mV with fast
activating and inactivating inward currents (Figure 2 B). These currents activated at potentials more positive than −30 mV (potential of the half maximal activation was −14.0 ± 0.7; n = 19) and reached maximal current amplitudes at +10 mV (Figure 2 D). The current peak was reached after 4.7 ± 0.6 ms (n = 16) which corresponds to activation time constant of 0.2 ± 0.06 ms (n = 6). The maximal current density of these currents was 5.7 ± 0.9 pA/pF (n = 16). Application of the L-type Ca2+ channel blocker nifedipine at a concentration of 10 μM reduced the currents to 34.7 ± 4.6 % (n = 5; p < 0.00001) of the control value observed before nifedipine application (Figure 2 C). Nifedipine did neither change the voltage-dependence of the currents (potential of the half maximal activation in the presence of 10 μM nifedipine: −16.0 ± 2.5 mV; n = 3) nor the activation kinetics (time to peak 10 μM nifedipine: 5.16 ± 0.8 ms; n = 3). Western-blot analysis of membrane proteins from RPE-J cells indicated that RPEJ cells express Cav1.3 Ca2+ channel subunits (Figure 2 E). Thus, RPE-J cells express L-type channels of the neuroendocrine subtype but not of the cardiac subtype. The expression of Cav1.3 subunits was confirmed by RT-PCR analysis which revealed the expression of a splicing variant including the exons 11, 32 and 44 (Figure 1F).

**Transient expression of wild-type bestrophin-1**

After transfection of RPE-J cells with WT-bestrophin-1 or the two mutants W93C and R218C, these different bestrophins were found to be localised in the cell membrane (Figure 3A). Expression of eGFP alone in RPE-J cells did not affect either voltage-dependence nor Ba2+ current kinetics (Table 1). Co-expression of eGFP together with wild-type bestrophin-1 led to faster activation kinetics (Figure 3B,C) measured as time-to-peak
(p < 0.0002; Figure 4B, 6A). In addition, the voltage-dependent activation of Ba$^{2+}$ currents in bestrophin-1 and eGFP co-transfected cells was shifted to more negative values (p < 0.00001; Table 1, Figure 4A). The membrane localisation of the different bestrophins was verified by biotylinisation assay (Figure 3 A). Comparable observations were made when the eGFP-bestrophin-1 fusion construct was used (3 D, E). This construct enabled us to verify that the above described changes in L-type channel activity were observed in cells showing the bestrophin-1 in the cell membrane (Figure 3D). In these cells bestrophin-1 transfection led to faster activation kinetics as well as to a shift of the voltage-dependent activation to more negative values (Figure 6). In both sets of experiments bestrophin-1 did not alter L-type channel amplitude, the maximal current density remained unchanged (Table 1). Application of nifedipine (10 $\mu$M) reduced the Ba$^{2+}$ currents in transfected cells to 33.0 $\pm$ 4.3 % (n = 4) of the control amplitude observed before application of nifedipine. Faster activation kinetics were observed at all membrane potentials at which Ca$^{2+}$ channel currents were measured (Figure 4B). Thus, the presence of wildtype bestrophin-1 led to modulation of L-type Ca$^{2+}$ channel activity in RPE cells by acceleration of the activation kinetics and shift of the voltage-dependence to a more negative voltage-range.

Effects of mutant bestrophin-1

In this study we concentrated on the two most frequently described mutants of bestrophin-1 associated with Best's disease, W93C and R218C (www.uni-wuerzburg.de/humangenetics/vmd2.html). In our first set of experiments effects of the mutant W93C were investigated (Figure 5B). In RPE-J cells transfected with W93C-bestrophin activation and inactivation kinetics were several fold slower than in non-transfected cells (p < 0.0003; Figure 5A,B) or
cells transfected with wild-type bestrophin-1 (p < 0.0002; Figure 5A, B). However, the shift in the voltage-dependent activation to more negative values was comparable to that observed with wild-type bestrophin-1 (Figure 5D). Due to the slow inactivation in the presence of W93C bestrophin, after 50 ms a rest steady state current of 40 % of the maximal current amplitude remained in cell transfected with W93C bestrophin which is 3 fold higher than in cells transfected with wild-type bestrophin-1 or in untransfected cells (p < 0.00005). The mutant R218C had different effects on L-type channel activity (Figure 5C). RPE-J cells transfected with R218C bestrophin showed L-type Ba²⁺ currents with voltage-dependent activation (Figure 5D) and activation kinetics comparable to cells which have been transfected with wild-type bestrophin-1 (Figure 5A). However, this mutant influenced L-type channel inactivation (Figure 5C). In cells transfected with R218C the currents inactivated significantly faster than with wild-type bestrophin-1 (p < 0.02) or in untransfected cells (p < 0.001). In contrast to control cells or cells transfected with wild-type bestrophin-1, L-type channels in RPE-J cells transfected with R218C bestrophin inactivate completely (Table 1). Both mutant bestrophins affected another characteristic of L-type Ca²⁺ channels. RPE-J cells transfected with either R218C or the W93C mutants showed a severe run-down of L-type channel activity in the perforated-patch configuration (Figure 5E).
Discussion

Bestrophin-1, the product of the VMD2 gene, is expressed only in RPE cells. This is the first study of bestrophin-1 that has thoroughly characterized the effects of bestrophin-1 on ion transport in an RPE derived cell line. To date, all previous studies have almost completely relied on data derived from exogenous expression in HEK 293 cells. Of significant interest, we find that when expressed in RPE derived cells, bestrophin-1 altered the characteristics of L-type Ca\textsuperscript{2+} channel activity, suggesting that bestrophin-1 plays a role in regulation of Ca\textsuperscript{2+} entry via L-type Ca\textsuperscript{2+} channels.

Systemic application of the L-type channel blocker nimodipine did not affect the ERG a- and b- waves which arise from activation of photoreceptors in the neural retina. This is in accordance with other ERG studies using dihydropyridine compounds to inhibit L-type channels. However, nimodipine selectively affected an RPE generated ERG component, the light-peak. It is likely that nimodipine cannot pass through the blood/retina barrier and affects only L-type channels in the RPE. In addition, the c-wave, another signal which originates in the RPE, is unchanged. However, this is likely because the c-wave results from hyperpolarisation of the apical membrane (27). L-type Ca\textsuperscript{2+} channels are activated by depolarisation. In summary, in vivo the RPE require L-type Ca\textsuperscript{2+} channels to properly generate the light-peak. Furthermore, this is the first demonstration that L-type Ca\textsuperscript{2+} channels play a functional role in the RPE of an intact eye.

Depolarisation of RPE-J cells in the presence of extracellular Ba\textsuperscript{2+} led to activation of voltage- and time-dependent inward currents. By means of voltage-dependence and sensitivity to the dihydropyridine nifedipine these currents could be identified as currents through L-type Ca\textsuperscript{2+} channels (45, 46). This observation is in accordance with investigations
of cultured or freshly isolated RPE cells from various species (47-53). The dihydropyridine nifedipine did neither change activation kinetics nor voltage-dependence. Thus, it is likely that a main portion of the current is carried by L-type channels. The currents showed fast activation kinetics, relatively negative activation thresholds and a rather low dihydropyridine sensitivity. With an activation time constant of 0.2 ms and a potential of half maximal activation at $-14\text{mV}$ currents through L-type channels in RPE cells are comparable with currents of Cav1.3 Ca$_{2+}$ channel subunits in heterologous expression studies (42, 54, 55) and native cells (56). In the presence of nifedipine a rest current of about 40 % of the control current was measured. This seems to be a difference to Cav1.3 channels in the expression system (42) but seems to be characteristic for these channels in native cells (56). However, the currents in RPE cells show fast inactivation compared to Cav1.3 currents in heterologous expression or in native cells. This might be a characteristic of the splicing variant expressed in RPE cells (57). So far, this variant has not been published and its characteristics are unknown. The expression of these Ca$_{2+}$ channel subunits in RPE-J cells was confirmed by Western-blot analysis. Here, Cav1.3 could be detected but not Cav1.2. The expression of the skeletal subtype or the photoreceptor specific subtype is unlikely (46, 58). In studies of human or rat cells this Ca$_{2+}$ channel subunit appeared as the predominant Ca$_{2+}$ channel subunit in the RPE (51-53). Thus, the L-type channels in RPE cells are predominantly L-type channels of the neuroendocrine subtype.

Using a biotylinisation assay, we demonstrated that heterologously expressed bestrophin-1, the mutant W93C and the mutant R218C are localised in the cell membrane. RPE-J cells transfected with wild-type bestrophin-1 showed L-type channel currents with different
characteristics. The current density was comparable to that of untransfected cells but activated at more negative potentials and showed faster activation kinetics. The faster activation kinetics could be observed at all potentials at which Ca\textsuperscript{2+} channels were active. Thus, the changes in activation kinetics were not due to the shift in the voltage-dependent activation. These effects were seen when bestrophin-1 was either co-expressed with eGFP as reporter or when an eGFP-bestrophin-1 fusion construct was used. eGFP alone had no effects on L-type channel activity. Furthermore, the eGFP-bestrophin-1 fusion construct permitted us to correlate the bestrophin-1 localisation with effects on L-type channel activity. The effects we describe here were observed in cells where bestrophin-1 was localised at the plasma membrane. Based on these data we propose that the presence of bestrophin-1 results in specific changes in the activity of L-type channels. This hypothesis is supported by the investigation of the effects of mutant bestrophins. Both mutants investigated had no effects on the current density but shifted the voltage-dependent activation to more negative values similar to that in cells transfected with wild-type bestrophin-1. However, both the R218C and W93C mutants demonstrated different effects on L-type channels kinetics. W93C slowed down both activation and inactivation kinetics whereas R218C only affected the inactivation kinetics. Thus, bestrophin-1 affects L-type channel activation kinetics and voltage-dependence. The mechanism underlying these effects requires further evaluation. The observed bestrophin-dependent changes in the L-type channel characteristics are comparable to those known from Ca\textsuperscript{2+} channel β-subunits (45, 46) or from phosphorylation by protein kinases (45, 46, 50-52). The latter mechanism is supported by the observation that in cells transfected with mutant bestrophins L-type channels show an accelerated run-down of Ca\textsuperscript{2+} channel activity.
One effect of bestrophin-1 expression which was identical regardless of mutation, was a shift of voltage-dependent activation to more negative values, closer to the approximate resting potential of RPE cells. This shift may serve to enable L-type channels in RPE cells to contribute to rises in intracellular free Ca\textsuperscript{2+} as second-messenger at rather negative membrane potentials (40), close to the resting potential of RPE cells of –40 to –45 mV (26, 27). This is a property which has been described for Ca\textsuperscript{2+} channels of Cav1.3 subtype (42) and bestrophin-1 might facilitate this. Furthermore, prior studies on the regulation of L-type channels in the RPE have suggested that modulation of the voltage-dependence to increase the number of active channels near the resting potential of RPE cells may be more important to generate rises in intracellular free Ca\textsuperscript{2+} than depolarisation of RPE cells (40). Failure to properly regulate the exchange of Ca\textsuperscript{2+} resulting from these responses could lead to uncontrolled changes in Ca\textsuperscript{2+} homeostasis.

Best’s vitelliform macular dystrophy is associated with a decrease of the light-peak in patient’s EOG (1). Systemic inhibition of L-type channels reduced the light-peak amplitude. Second-messenger dependent activation of L-type channels increases intracellular free Ca\textsuperscript{2+} (40) and subsequently activates Ca\textsuperscript{2+}-dependent Cl- channels (28). Thus, activation of L-type Ca\textsuperscript{2+} channels likely contributes to the generation of the light-peak peak conductance. The bestrophin-dependent changes in L-type channel properties must lead to corresponding changes in the light-peak. Transfection of the rat RPE with wild-type bestrophin-1 in an animal model for Best’s disease did not change the light-peak amplitude but accelerated the rise of the light-peak (35). The mutant W93C led to slower rises in the light-peak and the mutant R218C had the same effect on the rise of the light-peak as wild-type bestrophin-1 but the light-peak amplitude was much smaller (35).
In previous publications bestrophin-1 was described as a Ca$_{2+}$-dependent Cl$^-$ channel (17, 21). Using site-directed mutagenesis properties of the channel pore of bestrophin-2 were characterised. However, recent reviews state that the picture of bestrophin as a Cl$^-$ channel is not complete (59, 60). We found that the expression of bestrophin-1 leads to changes in the activity of L-type Ca$_{2+}$ channels. These functions are not mutually exclusive and it is possible that bestrophin might combine the function as Cl$^-$ channel and Ca$_{2+}$ channel regulator and brings both ion channel types into synergistic function. For instance, the combination of these functions could establish a direct feed-back loop for activation of Ca$_{2+}$-dependent Cl$^-$ channel in the presence of L-type channels. Our data imply disturbed Ca$_{2+}$ homeostasis as an additional factor in the chain of events leading Best’s macular dystrophy. Thus, the effects on Ca$_{2+}$ channel activity might open new ways to understand the heterogeneity between onset of macular degeneration and reduction of the light-peak in Best’s macular dystrophy (4-6, 9, 12, 29, 30, 32-34, 61).

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**Figure Legends**

**Figure 1: Effect of the L-type Ca\textsuperscript{2+} channel blocker nimodipine on light-peak amplitude**

The effect of nimodipine on the conventional ERG (A, C, D) and DC-ERG (B, E) was determined 30 min after intraperitoneal injection of a 1mg/kg dose of nimodipine in rats. Nimodipine (n = 6) had no effect on the conventional ERG waveform (A) compared to control animals receiving vehicle alone (n = 5). This was confirmed by analysis of a- and b-wave amplitude (C) and implicit time (D) in response to a series of flashes of increasing intensity. DC-ERGs were recorded to a single flash at 2.7 log cd m\textsuperscript{-2}. The mean waveforms generated from animals receiving nimodipine (n = 8) or vehicle alone (n = 8) are shown in D. Note the general reduction in amplitude of the DC-ERG waveforms in animals receiving nimodipine. LP amplitudes are shown in E. Bars represent mean LP amplitudes. Individual LP amplitudes for each group are superimposed over the bars in E. Mean isolated LPs collected between 110 sec and 300 sec were normalized to the 110 sec data point and are presented in E.

**Figure 2: L-type Ca\textsuperscript{2+} channels in RPE-J cells**

2A: Pattern of electrical stimulation to activate voltage-dependent currents in RPE-J cells. From a holding potential of –70 mV the cells were stepwise depolarised: 9 voltage-steps of 50 ms duration, + 10 mV increment. 2B: Inward currents activated by the electrical stimulation shown in 2A in the presence of 10 mM Ba\textsuperscript{2+}. 3C: Effect of the dihydropyridine nifedipine: Currents were activated by a voltage-step from –70 mV to +10 mV in the absence (control) and in the presence of nifedipine (10\mu M). 2D: Current/voltage plot of the data shown in 2B. Maximal current amplitudes were plotted against the potentials of the electrical
stimulation shown in 2A. 2E: Western-blots of membrane proteins from RPE-J cells and rat brain as control were stained for $\alpha_{1D}$ subunits (Cav1.3 subunits; left panel), for $\alpha_{1D}$ subunits in the presence of the corresponding blocking peptide (BP) and for $\alpha_{1C}$ subunits (Cav1.2 subunits; right panel). 2F: Data of RT-PCR analysis of Cav1.3 expression in RPE cells.

**Figure 3: Effect of wild-type bestrophin-1 transfection**

3A: RPE-J cells were co-transfected with vectors encoding GFP and one of the following: empty vector (sham), bestrophin-1 (wt), bestrophin-W93C (W93C), or bestrophin R218C (R218C). After 24hrs, cells were surface biotinylated. Following lysis, bestrophin or GFP were immunoprecipitated as indicated and blotted for biotin using streptavidin, for bestrophin using an anti-Best-1 monoclonal antibody, or for GFP using an anti-GFP monoclonal antibody. Note that wt, W93C, and R218C forms of bestrophin were biotinylated, and so present on the cell surface in contrast to GFP, an intracellular protein. 3B: Currents activated by the electrical stimulation shown in 2A; cell is transfected with bestrophin-1 and eGFP as reporter. 3C: Direct comparison of kinetics of L-type currents between a cell transfected with eGFP alone and a cell co-transfected with eGFP + bestrophin-1. Currents were normalized to the maximal current amplitude to allow a direct comparison. 3D: Fluorescence microscopy: RPE-J cell transfected with eGFP-bestrophin-1 fusion construct showing bestrophin-1 localisation in the cell membrane. 3E: Currents activated by the electrical stimulation shown in 2A; cell transfected with the eGFP-bestrophin-1 fusion construct. Recording derived from a cell showing a bestrophin-1 localization in the cell membrane.
Figure 4: Changes of voltage-dependent Ca\textsubscript{2+}-channel characteristics by bestrophin-1 transfection

4A: Curves of voltage-dependent activation. Curves were fitted using the Boltzmann equation to evaluate V\textsubbox{1/2} of the voltage-dependent activation. 4B: Time to peak values at membrane potentials at which the Ca\textsubscript{2+}-channels are active. Note: due to shift in voltage-dependent activation currents could be recorded over a larger voltage range in bestrophin-1 transfected cells. (nontransfected: n = 6; wt bestrophin-1 transfected n = 4). 4C: Inactivation time constants at membrane potentials at which the Ca\textsubscript{2+}-channels are active. Note: due to shift in voltage-dependent activation currents could be recorded over a larger voltage range in bestrophin-1 transfected cells. (nontransfected: n = 6; wt bestrophin-1 transfected n = 4).

Figure 5: Effect of mutant bestrophins

5A: For comparison, currents activated by the electrical stimulation shown in 2A in a cell transfected with wild-type bestrophin-1. 5B: Currents activated by the electrical stimulation shown in 2A in a cell transfected with the mutant W93C bestrophin-1. 5C: Currents activated by the electrical stimulation shown in 2A in a cell transfected with the mutant R218C bestrophin-1. 5D: Curves of voltage-dependent activation of L-type currents in cells transfected with either wild-type bestrophin-1, W93C-bestrophin-1 or R218C-bestrophin-1. 5E: Run-down of L-type currents in cells transfected with mutant bestrophin-1: Maximal current amplitude (normalized to the amplitude which has been measured directly after establishment of the perforated-patch configuration) was plotted over the experiment time. Membrane resistance and membrane capacitance remained unchanged during this time. L-type currents in cells transfected with wild-type bestrophin-1 (circles) were stable for more
than 10 min; with either W93C (triangles) or R218C bestrophin (squares) the currents were stable for only 2-3 min and then showed severe run-down. The most instable recording was with R218C.

**Figure 6: Comparison of kinetic parameters**

6A: Comparison of time-to-peak values as measure of the time-dependent activation of L-type currents in non-transfected cells or cells transfected with different bestrophins. 6B: Comparison of inactivation-time constants of L-type currents in non-transfected cells or cells transfected with different bestrophins. 6C: Comparison of rest steady-state of L-type currents in percent to the maximal current amplitude in non-transfected cells or cells transfected with different bestrophins. (asterisks indicate level of significance compared to untransfected or GFP transfected cells. Other significances: time-to-peak: WTbestrophin+eGFP/WTbestrophineGFP versus R218C = n.s.; WTbestrophin+eGFP/WTbestrophineGFP versus W93C = ***; inactivation time constant: WTbestrophin versus R128C = *; WTbestrophin versus W93C = ***; rest current amplitude: WTbestrophin versus W93C = ***; WTbestrophin versus R218C = *)

**Table 1**

Overview of all L-type channel characteristics in either non-transfected cells or in cells transfected with different bestrophins. Maximal current density, inactivation time constant ($\tau$), time-to-peak and rest current amplitude were estimated from currents activated by a step of the membrane potential from –70 mV to +10 mV. Data are given as mean ± SEM.
Figure 1

A. ERG

B. DC-ERG

C. Nimodipine (n=6) vs Control (n=5)

D. Nimodipine (n=6) vs Control (n=5)

E. LP Amplitude (% of max.)

n=8  n=8
Figure 2

A

B

C

D

E

F

Exon 11

450 Bp
390 Bp

Exon 32

365 Bp
322 Bp

Exon 44

254 Bp
227 Bp

RPEJ cells

Ca\textsubscript{\textalpha.3}

Ca\textsubscript{\textalpha.3} +
BP

Ca\textsubscript{\textalpha.2}

rat brain

Ca\textsubscript{\textalpha.3}

Ca\textsubscript{\textalpha.3} +
BP

Ca\textsubscript{\textalpha.2}
Figure 3

A

BWT Bestrophin + GFP
cotransfected cell

C

WT Bestrophin

D

WT Bestrophin-GFP
transfected cell
Figure 4

A

![Graph showing relative current (I/I_max) vs. membrane potential (mV) for nontransfected and WT Bestrophin.]

B

![Graph showing time to peak vs. membrane potential (mV) for nontransfected and WT Bestrophin.]

C

![Graph showing inactivation time constant vs. membrane potential (mV) for nontransfected and WT Bestrophin.]
Figure 5

A  WT Bestrophin

B  W93C Bestrophin

C  R218C Bestrophin

D  

relative current ($I / I_{\text{max}}$)

membrane potential (mV)

-80  -60  -40  -20  0  20

-0.2  0.0  0.2  0.4  0.6  0.8  1.0  1.2

WT  W93C  R218C

E  

relative current ($I / I_{\text{max}}$)

time / min

0  2  4  6  8  10  12

WT  W93C  R218C
## Table 1

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<tr>
<td><strong>current density pA/pF</strong></td>
<td>5.7 ± 0.9</td>
<td>4.1 ± 1.3</td>
<td>4.0 ± 0.4</td>
<td>3.8 ± 0.7</td>
<td>2.6 ± 0.6</td>
<td>4.0 ± 0.6</td>
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<td>n=12</td>
<td>n=6</td>
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<td>n=6</td>
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<tr>
<td><strong>inactivation time constant τ / ms</strong></td>
<td>12.0 ± 1.2</td>
<td>16.6 ± 7.2</td>
<td>7.8 ± 2.8</td>
<td>4.0 ± 1.1</td>
<td>31.7 ± 9.3</td>
<td>3.8 ± 1.2</td>
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<tr>
<td><strong>time to peak / ms</strong></td>
<td>4.7 ± 0.6</td>
<td>3.4 ± 0.8</td>
<td>1.3 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>8.3 ± 3.4</td>
<td>1.2 ± 0.4</td>
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<tr>
<td><strong>I_{rest} %</strong></td>
<td>9.5 ± 1.4</td>
<td>16.3 ± 7.2</td>
<td>15.7 ± 4.8</td>
<td>14.5 ± 3.8</td>
<td>41.0 ± 11.5</td>
<td>4.8 ± 1.4</td>
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<tr>
<td><strong>V_{1/2} mV</strong></td>
<td>-14.0 ± 0.7</td>
<td>-13.8 ± 1.8</td>
<td>-22.3 ± 1.6</td>
<td>-25.2 ± 2.4</td>
<td>-30.0 ± 2.5</td>
<td>-22.7 ± 2.2</td>
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