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The electro-oculogram

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Abstract

The retinal pigment epithelium (RPE) lying distal to the retina regulates the extracellular environment and provides metabolic support to the outer retina. RPE abnormalities are closely associated with retinal death and it has been claimed several of the most important diseases causing blindness are degenerations of the RPE. Therefore, the study of the RPE is important in Ophthalmology. Although visualisation of the RPE is part of clinical investigations, there are a limited number of methods which have been used to investigate RPE function. One of the most important is a study of the current generated by the RPE. In this it is similar to other secretory epithelia. The RPE current is large and varies as retinal activity alters. It is also affected by drugs and disease. The RPE currents can be studied in cell culture, in animal experimentation but also in clinical situations. The object of this review is to summarise this work, to relate it to the molecular membrane mechanisms of the RPE and to possible mechanisms of disease states.
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Abbreviations: ARMD, Age-related macular degeneration; AZOOR, Acute zonal occult outer retinopathy; CaCC, Calcium-activated Cl^- channel; CF, Cystic fibrosis; CFTR, Cystic fibrosis transmembrane conductance regulator; cAMP, Cyclic adenosine monophosphate; cGMP, Cyclic guanosine monophosphate; DAG, Diacylglycerol; DIDS, 4, 4′-diisothiocyanostilbene-2, 2′-disulphonic acid; DPC, Diphenylamine-2-carboxylic acid; DR:LT, Dark rise: light trough; ER, Endoplasmic reticulum; ERCA, Endoplasmic reticulum calcium ATPase pump; EOG, Electro-oculogram; ERG, Electroretinogram; ERP, Early receptor potential; FO, Fast oscillation; IP_3, Inositol 1, 4, 5-triphosphate; IP_3-R, the IP_3 receptor; ISCEV, International Society for Clinical Electrophysiology of Vision; NCX, Sodium–calcium exchanger; NBD, Nucleotide binding domain; PMCA, Plasma membrane calcium ATPase pump; PKA(C), Protein kinase A(C); PTK, Protein tyrosine kinase; RCS, Royal College of Surgeon's; RP, Retinitis pigmentosa; RPE, Retinal pigment epithelium; TEP, Trans-epithelial potential; TER, Trans-epithelial resistance; V_M; Membrane voltage

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1. Introduction

The retinal pigmented epithelium (RPE) is an electrically polarised pigmented epithelial monolayer that lies posterior to the photoreceptors where it plays a central role in maintaining the outer retina (Strauss, 2005). The RPE's functions include transporting retinol to the outer segments, phagocytosing shed photoreceptor outer segments (Besharse and Defoe, 1998) the transport of fluid and metabolites, and the regulation of the contents of the RPE cytosol and the subretinal space (Adorante and Miller, 1990; Bialek and Miller, 1994; Bialek et al., 1996; Lin et al., 1992; Rymer et al., 2001). Furthermore, the RPE forms a part of the outer blood retinal barrier by virtue of its tight-junctional complexes. It contains a number of potential drug transporters that are of medical interest and also excludes potentially harmful xenobiotics from the subretinal space (Kennedy and Mangini, 2002; Steuer et al., 2005). The RPE is responsible for the corneo-fundal standing potential (Kühne and Steiner, 1881) and the change in this voltage across the human eye evoked by change of illumination forms the basis of the clinical electro-oculogram (EOG) (Arden and Kelsey, 1962a; Kris, 1958). The light-EOG has also been recorded in other species: lizard (Griff and Steinberg, 1982), chicken (Gallemore et al., 1988), mouse (Kikawada, 1968), rat (Arden and Ikeda, 1964), rabbit (Ogawa, 1967), cat (Linsenmeier and Steinberg, 1982), primate (Valeton and van Norren, 1982). The light rise of the EOG results from the release of a substance, termed the "light rise" or "light peak" substance from the photoreceptors (the chemical composition of which is unknown) that interacts either directly or indirectly with the RPE (Gallemore et al., 1988). There is indirect evidence (see Section 9) that this substance causes an increase in the intracellular concentration of calcium ($[Ca^{2+}]_{in}$) within the RPE which in turn opens a basolateral ionic Cl^- channel, thus depolarising the basal membrane (Gallemore et al., 1988, 1993; Gallemore and Steinberg, 1989b) and leading to the characteristic light rise of the EOG (Gallemore and Steinberg, 1993). However, the exact nature of the basolateral Cl^- channel has not been determined, but is believed to be bestrophin (Hartzell et al., 2005b; Marmorstein et al., 2004; Strauss and Rosenthal, 2005).

The light rise of the light-EOG is a slow complex change. Following dark adaptation, the return to light causes an increase in the ocular standing potential that peaks at ~7–10 min. Following this the voltage decreases to a trough at ~22 min. Smaller and slower ripples occur for over 2 h. The amplitude of the rise is dependent upon the

duration of the previous period in darkness (see Section 13) up to a duration of 22 min. This is not the only effect of light on the RPE. When the period of light and dark intervals is ~1 min an induced oscillation—the fast oscillation (FO) of the standing potential occurs at the stimulus frequency. The FO has an opposite sense to the light rise. Immediately following retinal illumination there is a fall in the standing potential and a light trough develops that begins to rise ~30 s after light onset. The FOs are believed to be a function of the alteration of basolateral Cl^- channel transport following a fall in subretinal $[K^+]_{out}$ (Blaug et al., 2003). The FOs provides insights into inherited dystrophies (Weleber, 1989) and potentially RPE function as well (Schneck et al., 2000).

Whilst the EOG provides useful information about the integrity of the photoreceptor-RPE complex in acquired and inherited retinal degenerations, the test in itself is not specific for RPE function. Therefore, investigators have employed alternative means to detect early changes in RPE physiology. The EOG changes in response to acetazolamide (Kawasaki et al., 1986), mannitol (Kawasaki et al., 1977; Shirao and Steinberg, 1987) hypoxia and hyperoxia (Marmor et al., 1985) have all been explored as potentially suitable non-photic stimuli that directly affect the RPE. In man, alcohol apparently initiates the same voltage changes and series of intracellular events that cause the light-induced change in the RPE (Arden and Wolf, 2000a; Skoog et al., 1975) and may provide an additional method of detecting early RPE dysfunction (Arden and Wolf, 2003; Arden et al., 2000). Alcohol-induced changes similar to those seen in man have been observed in dark-adapted sheep in vivo (Knave et al., 1974) and alcohol changes the voltage (in vitro) of light-adapted bovine RPE (Pautler, 1994), but the mechanisms remain unclear.

Our intention is to provide a background for the understanding of the ionic channels within the RPE and how alterations in them cause the changes in RPE membrane potential that produce the clinical EOG currents. To this end we discuss the basic biophysics of cell membranes and the electrophysiological techniques of measuring the ionic currents. In addition we will review practical aspects of the EOG, because as a non-invasive technique it can be used to investigate not only membrane properties of the RPE, but also the functioning of the subretinal space and photoreceptors in disease states and can provide information unrelated to changes of visual sensation and supplemental to those of modern imaging techniques. For these reasons, the EOG is a tool often used in clinical research on the retina.

2. Discovery and the first analyses

The potential voltage difference that occurs between the cornea and fundus was discovered by Du Bois Reymond (1849). He showed that it persisted for long periods in the isolated eye. Kühne and Steiner (1881) and de Haas (1901) also measured the voltages after removing successively cornea, iris, lens vitreous and retina. Only when the RPE had been damaged did the potential vanish, and this localised the source of the current production to the RPE. Although illumination was known to affect the potential recorded between the cornea and fundus (Himstedt and Nagel, 1902) the capillary electrometers used in early work were not sufficiently sensitive or stable to analyse the changes in detail (Einthoven, 1893). With the advent of electronic amplification, the sensitivity problem was solved, but the amplifiers were unstable, and required condenser-coupling, so slow changes were not amplified. In the 1940s, Noell was able to employ systems both stable and sensitive (DC amplification) and could follow the slow changes; he related the c-wave of the electroretinogram (ERG) to the later and still slower responses. He used poisons that selectively damaged the RPE, as demonstrated by histological changes caused. He found that azide, acting on the RPE transiently increased the "standing potential" and the c-wave of the ERG, while iodate, that damaged the RPE selectively, not only caused a fall in the standing potential but also reduced the azide increase, and reduced the c-wave. Faster changes caused by illumination—i.e. other components of the ERG—were less affected (Noell, 1942, 1952, 1953a, b).

3. Development of current fields round the eyes

The RPE consists of a layer of cells connected by tight junctions, so that the resistance to current flowing across the membrane in the spaces between the cells (the paracellular resistance) is 10 times greater than the transmembrane resistance itself (Brindley and Hamasaki, 1963; Miller and Steinberg, 1977a, b). Therefore, voltage developed across the RPE implies that at its origin current will flow at each point normal to the surface, and the return paths are through the paracellular resistance. Because the RPE follows the curvature of the globe, the current flow at each point can be split (formally) into three vectors. One is in the optic axis, and two at other axes at right angles to it, pointing medially or vertically. At the fundus, nearly all the current flows in the radial vector (in the optic axis). At other positions, there will be current vectors flowing in the optic axis and also vectors in the lateral, medial, superior, and inferior directions will be developed. Because of the approximately spherical shape of the eye non-radial current vectors will approximately cancel. For example, in the nasal part of the RPE, there will be a temporally directed vector, which will cancel with the nasally directed vector from the temporal part of the RPE. Hence the net current flow will be due to the vector in the optic axis and appears to be a dipole in the optic axis, with the cornea positive and the fundus negative. This explains the name given to the potential recorded across the eye, the corneo-fundal potential. The current flowing from such a dipole will spread symmetrically in all directions about the optic

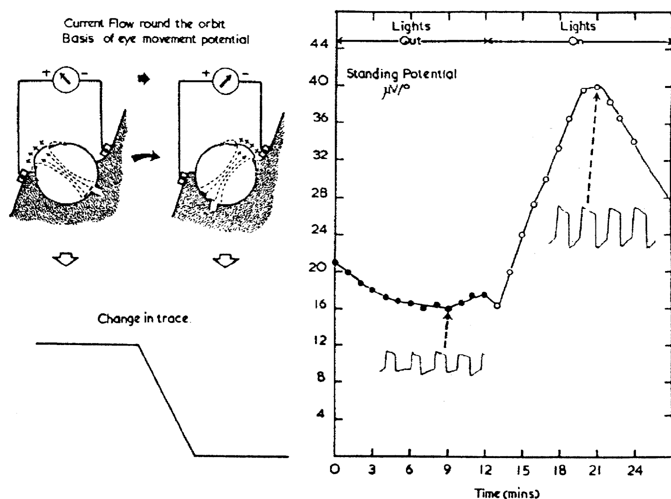

Fig. 1. The generation of the light rise by the corneo-fundal standing potential. From Arden (1962).

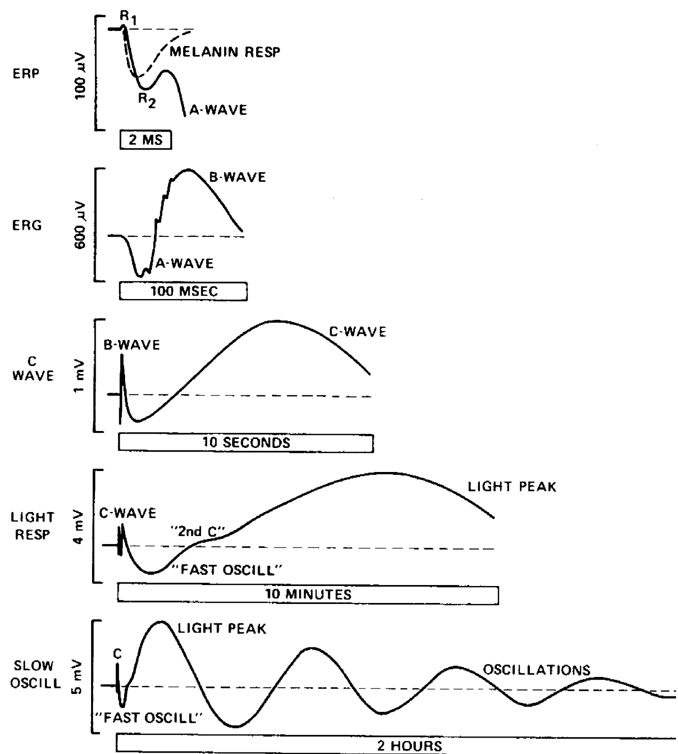

Fig. 2. Voltage changes resulting from photic stimulation of the eye recorded using different timescales, as indicated on each trace. The early receptor potential (ERP) is produced by a conformational change in the outer limb membrane that occurs when visual pigments absorb light. The ERG a- and b-waves are produced mainly by changes in photoreceptor dark current and the hyperpolarising rod bipolars. The c-wave is the result of the RPE's apical membrane hyperpolarisation in response to a decrease in subretinal $[K^+]$ and occurs after the faster ERG components generated in the neural retina. The fast oscillation trough occurs approximately 30 s after light onset and is due to the RPE's basal membrane hyperpolarising. The slow oscillation that is recorded as the EOG peaks at ~8 min is due to the RPE basal membrane depolarising. After the peak of the light rise, the transepithelial potential continues to fluctuate for several hours but these changes have not been investigated systematically. Printed with permission of Marmor and Lurie (1979).

axis. Therefore, as the eye moves, the voltages recorded between relatively distant skin electrodes will vary with the angle of rotation of the eye. This is shown in Fig. 1, which illustrates how the magnitude of the responses to fixed eye movements can be used to measure the magnitude of the current produce by the RPE. This simple view of the eye movement potential suggests that abnormalities in the posterior pole will cause larger changes to the current than changes near the equator and that recorded voltage will vary with the sine of the angle moved. However, neither of these suppositions is correct. One reason for this is that the cells of the RPE (which generate the voltage) form a syncitium so damage to some of the RPE cell causes similar voltage changes over a large area. Another is that for the relatively small displacements associated with rapid saccades, it is difficult to be certain whether a linear or sine relation holds between voltage change and angular displacement. The ease with which eye motion could be recorded led to attempts to use the EOG for eye-movement recordings. However, difficulties were found in calibrating such a system, because the apparent magnitude of the dipole was not constant (Aserinsky, 1955; Francois et al., 1955; Kolder, 1959; Miles, 1940; Taumer et al., 1974; ten Doesschate and ten Doesschate, 1955). It became apparent that one of the factors modifying the voltage was light. The first complete description of the human light–dark sequence was due to Kris (1958) but an analysis of the nature of the response and the recognition of its clinical utility is usually attributed to Arden (Arden, 1962; Arden and Barrada, 1962; Arden et al., 1962; Arden and Kelsey, 1962a) (Fig. 1) who showed that a small reduction in light intensity provoked a decrease in voltage, the dark trough, which was not related to the preceding light level, although the change from dark to light caused a transient rise of voltage (the light peak) the magnitude of which was linearly related to the logarithm of retinal illumination.

4. The full picture of the DC ERG

The full sequence of the voltage changes is shown in Fig. 2. The electroretinographic a- and b-waves occur in about 0.1 s: following this there is a slower c-wave. Following this sequence, there is a slow cornea-negative swing, the after negativity, followed by a "second c-wave" and then a still slower and larger increase of voltage, the "light rise". These changes have been observed in a number of different mammals. There are species differences. In man, even though light continues, this increase in the corneo-fundal potential is not maintained, but after a peak at 8 min sinks to a trough level at 22 min. If a light-adapted eye is placed in darkness, the potential falls to a level nearly identical to the lowest level reached at the 22–24 min trough. Following this trough, in light or in darkness, further slow rhythmic changes in voltage are seen that may persist for 2 h or more. For reviews on this topic see Marmor and Lurie (1979) and Steinberg et al. (1985).

5. Concepts of membrane voltage

Since the original description of the human EOG, work in animals, isolated eyes and isolated RPE preparations has resulted in a great deal of information about the nature of the ionic channels, cotransporters and pumps in the apical and basal surfaces of the RPE. Because the RPE is a secretory epithelium this knowledge is fundamental to our understanding of how fluid is transported across the epithelium. The development of the EOG likewise can be explained by the regulation or "gating" of these ionic channels by "second messengers"—pathways that link membrane mechanisms to intracellular changes. The methods employed include a variety of electrophysiological techniques that have each provided valuable insights into the membrane voltage (V_M) changes of the RPE, and the ionic channels involved in generating the clinical waveform of the EOG.

The V_M results from the difference in ionic concentrations across the plasma membrane and the relative permeability of the membrane to the ions in the intra and extracellular spaces. Alterations in the permeability or conductance of the membrane to the ions by the gating of ionic channels leads to a change in V_M as ionic currents pass across the membrane. Therefore, we will discuss the basic biophysics of ions and ionic channels and the forces that are involved in generating ionic currents that lead to changes in V_M. For a more detailed account of these underlying principals see the following texts Hille (2001), Sakmann and Neher (1995) from which the following section is summarised.

5.1. *The passage of ions through membranes and the membrane potential*

The phospholipid bilayer of the cell membrane is practically impermeable to ions; therefore any passage of ions across the membrane occurs in small specialised regions, via protein-containing ionic channels, pumps and cotransporters. Many ionic channels are specifically shaped (and charged) to permit the selective passage of a few types of ions, which will tend to move from a region of high concentration to one of a lower concentration (i.e. down their concentration gradient).[1]

Since ions are electrically charged, their movement through ionic channels causes a change in the charge across the lipid bilayer. For example, as positively charged potassium ions move out of a cell, the interior becomes negatively charged with respect to the exterior and so V_M hyperpolarises. This change in voltage eventually opposes the movement of the ions and an equilibrium is established approximating the Nernst equilibrium potential (Nernst, 1888) (see Eq. (1)).

$$E_X = RT/zF \ln\{[X]_{out}/[X]_{in}\}, \tag{1}$$

where E_X is the equilibrium potential (volts) of ion X; $[X]_{out}$ the concentration of ion X outside cell, $[X]_{in}$ is concentration of ion X inside the cell, R the gas constant ($8.314\,J\,K^{-1}\,mol^{-1}$), T the absolute temperature, F the Faraday's constant ($96{,}500\,C\,mol^{-1}$); z the valence of ion or charge, i.e.: $Cl^- = -1$, $Ca^{2+} = +2$.

Other physical properties of ionic channels as well as the membrane voltage will influence the resting V_M. Because potassium (K^+) channels have a 10^3 times higher permeability to K^+ than Na^+ (these are the only two cations present in relatively high concentration) and sodium channels in the resting state do not have a high permeability, the resting potential of cell membranes such as those of neurones approximates the potassium equilibrium potential. In the RPE the intracellular K^+ activity is $\sim$90 mM and the subretinal $[K^+]$ varies from 2–5 mM which would give a Nernst potential for V_{Apical} between -77 and -101 mM at 37 °C. In human RPE, V_{Apical} ranges from -35 to -63 mV have been reported (Quinn and Miller, 1992), showing that other ionic channels contribute to V_M.

Cell membranes are not simple permeable membranes. The ionic channels have properties that constrain ionic currents and maintain V_M by holding some ions away from equilibrium. It has been shown that single channels change their state frequently, opening and closing very rapidly. The conductance depends upon the proportion of time the channel remains in the same state. If the open state occurs most of the time, the particular ion involved can move toward its equilibrium potential. Therefore, stimuli which change the channel state are said to "gate" it. Opening of the ionic channels allows these ions to move towards their equilibrium potential thus generating ionic currents and a change in V_M.[2]

[1] Even though Na^+ ions have a smaller atomic radius than K^+ they do not pass easily through potassium channels (Doyle et al., 1998). Interactions within the pore between ions and amino acids determine the preferential permeability of one ion above another for each channel type. However, most Cl^- ionic channels are not as selective (Linsdell et al., 1997a) with some displaying cation permeability (Qu and Hartzell, 2000). The significance of permeability and conductance of an ion is different. The permeability of an ion relates to how easily an ion enters the pore whilst the conductance indicates how easily the ion traverses the channel. This depends upon the difference between the free energy of the ion in solution compared to that in the pore (Dawson et al., 1999; Smith et al., 1999).

[2] The behaviour of ions in a pore or channel may be complex. Ions are associated with water molecules, and may interact with each other, and also with charged amino-acids in the cell membrane surface proteins and the negative charge of the phospholipid bilayer all interact with the ions inside a cell and form stable complexes reducing their availability to traverse through an ion channel or pore. Therefore, predictions about ionic behaviour in a dilute solution differ to the actual situation and compensation can be made for some of these interactions by using the ionic "activities" instead of molar concentration (Blum, 1980; Debye and Hückel, 1923; Roberts and Stokes, 1965). Furthermore, when a large difference exists between the intra and extracellular concentrations of an ion such as Ca^{2+} where intracellular free concentrations are 10^3 times lower than extracellular levels (Feldman et al., 1991) the electrochemical gradient is unfavourable for Ca^{2+} to exit the cell and so the ionic current varies as a non linear function of V_M as defined by the

5.2. Maintenance of membrane potential

In the RPE, which is a secretory epithelium, the passage of a cation is often accompanied by the passage of an anion, so that apart from electrical current flow, net movement of salts, acids or alkalis develops and with it the movement of water is induced across the membrane. Any imbalance in such processes, if they continued indefinitely, would alter the composition of the cell. Therefore, there are various active and passive regulatory mechanisms that act to prevent long-term alterations in the cytosolic environment in addition to the mechanisms that transport water ions and other solutes across the epithelium.

One ubiquitous transporter in epithelia is the ouabain-inhibitable Na–K–ATPase pump that is present in the apical membrane of the RPE (Hu et al., 1994; Rizzolo, 1990). This pump utilises the energy of ATP hydrolysis to move three Na^+ ions out of the cell in exchange for two K^+ ions that enter. This pump is the main way in which the cytosol concentration is kept rich in potassium and low in sodium. It only contributes ~10 mV to V_{Apical} in the frog (Miller et al., 1978). The constant fluid elimination from the subretinal space results in an adhesive force between the RPE and retina. Inhibition of the Na–K–ATPase activity by ouabain reduces the adhesive forces between the tissues (Frambach et al., 1989). Even bicarbonate transport requires the activity of Na–K–ATPase because this pump establishes the sodium gradient which serves to uptake bicarbonate and thus establishes the driving forces for bicarbonate movement across the basolateral membrane. Numerous transporters have been localised in the RPE plasma membrane. They operate by coupling ions, amino acids or metabolites in exchange for another compound thereby regulating cell pH, osmolarity and cell volume, as well as transporting metabolic wastes or nutrients across the membrane (Adorante and Miller, 1990; Lin et al., 1992, 1994; Miller and Steinberg, 1977a, 1979; Peterson and Miller, 1995). One example is the bumetanide- and furosemide-inhibitable Na–K–2Cl cotransporter (la Cour et al., 1997; Xu et al., 1994), that regulates cell volume (Adorante and Miller, 1990) but does not contribute to the membrane potential as there is no net movement of charge across the membrane. For reviews see Flatman (2002) and Haas and Forbush (2000). Another very important function of this cotransporter is that it is the only means whereby Cl^- can be moved from subretinal space to the basal surface of the RPE. Sodium ions move with the Cl^-, and this accounts for most of the fluid flow. In contrast, an antiporter or exchanger, exchanges one ionic species for another across the membrane. Thus the Na^+–H^+ antiporter exchanges one Na^+ ion for a proton to counteract the electrogenic Na^+–HCO_3^- cotransporter which enables control of intracellular pH (Hughes et al., 1989; Lin and Miller, 1991).

A further mechanism of fluid transport is controlled by aquaporins of which 11 mammalian types have been identified. Their discovery is recent (Agre et al., 1993) and their regulation is complex. These channels may be associated with intracellular vacuoles, which "traffic" (i.e. move to and fuse with the membranes) under the influence of cyclic adenosine monophosphate (cAMP) or associated with Na–K–ATPase on the plasma membrane in the choroid plexus (Nielsen et al., 1993) to regulate water movement. Their properties in the RPE are not fully understood although they are essential for water transport (Ruiz and Bok, 1996; Stamer et al., 2003) (for review see King et al., 2004). More conventional ionic channels along with transporters also play a role in the movement of ions and water across membranes and the quantity per unit time varies, as the epithelium carries out its functions. The mechanisms by which specialised membrane proteins regulate the movement of charged ions and water are of interest, both on their own account and because of any change in relationship to disease. Because transport is associated with electrochemical phenomena, electrophysiology provides insights into these mechanisms.

V_M may also influence the conductance of an ionic channel so that the gating is voltage dependent. In this case, the ionic current will be zero when the equilibrium potential equals the membrane potential so that Ohm's law ($E = I/g$) can be rewritten (Eq. (3)) to account for the membrane potential.

$$I_X = g_X(V_M - E_X), \tag{3}$$

where I_X is the ionic current of ion X; g_X is the conductance of ion X across the membrane (reciprocal of resistance); V_M is the membrane potential and E_X is the Nernst equilibrium potential for ion X (Hodgkin et al., 1952).

This relationship holds when the current versus voltage (I–V) relation is linear and the intercept is at zero. The conductance (g) is dependent solely upon the difference in ionic concentrations across the membrane as described by the Nernst potential and is independent of V_M. However, for many RPE membrane channels the probability that the channel is open is greatly influenced by membrane voltage. Therefore, it is of interest to determine how these currents vary with V_M.

(*footnote continued*)
Goldman–Hodgkin–Katz current equation (Goldman, 1943; Hodgkin and Katz, 1949).

In practice, determining the equilibrium point for a complex system of ionic channels is handled well by the Goldman–Hodgkin–Katz voltage equation which states that at equilibrium the sum of all ionic currents will be zero. However, the permeability (P) of an ion is governed by the thickness of the membrane and the concentration difference of the ion as it passes through the membrane. Assuming then that ions act independently through the membrane and the electric field of the membrane is constant. Then the reversal potential (E_{rev}) is dependent upon the sum of the permeabilities multiplied by the Nernst potentials of the ions (Eq. (2))

$$E_{rev} = RT/zF \cdot \ln\left\{\sum_a^n [P \cdot ([X_i^+]_{out}/[X_i^+]_{in})] + \sum_a^n [P \cdot ([X_i^-]_{in}/[X_i^-]_{out})]\right\} \tag{2}$$

.

5.3. Voltage clamp and rectification

Although the original analysis of nerve fibre membrane activity was carried out with quite large electrodes in giant nerve fibres, the advent of intracellular recordings with glass micro-pipettes extended this to mammalian cells (Hodgkin and Huxley, 1952a–c; Hodgkin et al., 1952; Hodgkin and Katz, 1949; Ling and Gerard, 1949a–c). Further advances were made when a second electrode was placed within the cell so that V_M could be controlled and held constant by an external current (Cole, 1949; Marmont, 1949). Utilising this technique of voltage clamp, the membrane current could be plotted as a function of voltage.

The membrane current (I) was the sum of the ionic current (I_j) that depends upon the conductance of the ionic channels and the local capacity currents that occur when there is a change in the ionic density (charge) between the inner and outer surface of the membrane (Eq. (4)).

$$I = C_m(\delta V/\delta t) + I_j. \quad (4)$$

To isolate I_i the investigators inserted two electrodes into the axon. One electrode recorded the membrane potential whilst the other injected current into the axon to control the local V_M in a series of fixed steps. It was now possible to record I_i directly and determine the direction of I_i at holding potentials that depolarised (or hyperpolarised) the axon over a wide range—from its resting V_M to the reverse potential at the peak of the action potential. The V_M under these two conditions corresponded to the equilibrium potentials of K^+ and Na^+, respectively, and alteration of the intracellular or extracellular ionic composition confirmed that the resting and active membrane potentials changed as though the activity of these two ions controlled V_M under these two conditions (Hodgkin and Huxley, 1952a–c).

Such experiments cannot be carried out in the RPE without modifications, and the methods of analysis are discussed below. In practice, even in squid giant axon, there are technical difficulties, for example, when the voltage step begins, the current charges up membrane capacitances, and therefore a transient current is seen that requires nulling by a fast feedback amplifier.

6. Methods for analysing membrane mechanisms

6.1. The Ussing chamber

This consists of two independent half chambers which when joined seal an epithelium between them, creating an apical and basal bath in which recordings can be made of currents and voltage across the tissue (frog skin was the first to be investigated). The resistivity across the RPE is high (~2000 Ω cm^2) because tight junctions between cells create an obstruction to the passage of most ions between the cells (Joseph and Miller, 1991). Consequently, (almost) all current must flow through apical and basal surfaces of the epithelial cells. To record the trans-epithelial resistance (TER) a 2-s pulse of 4–10 mV is applied across the tissue. The instantaneous current (I) is recorded and Ohm's law applied to give the TER. There is also a trans-epithelial potential (TEP), because apical and basal membranes have differing voltages (due to the different ionic channels they contain) and the $TEP = V_{Basal} - V_{Apical}$ (Levi and Ussing, 1949). The advantages of using an Ussing chamber are that the RPE retains its cell–cell contacts and the interactions between the RPE and the retina may be explored in detail. With specific drugs, the intracellular signalling pathways can be modified so that a detailed picture of RPE physiology can be constructed. Hans Ussing introduced the short-circuit procedure in which the TEP was held at zero volts by applying a current. When the ionic composition of the fluid on both sides of the epithelium is identical and there is no net voltage difference across it, then the electrochemical gradient across the apical and basal membranes must be the same. Therefore, any current and voltage changes across the preparation (or at either the apical or basal membranes) must be due to the active electrogenic transporters (Ussing, 1953; Ussing and Zerahn, 1951; Voûte and Ussing, 1968) (Fig. 3). The short-circuit current is therefore an indication of the contribution of active processes (transporters) to ion and water movement. In contrast, the open-circuit current is the current that results from the electrochemical driving forces across ionic channels plus a contribution from the active transporters.

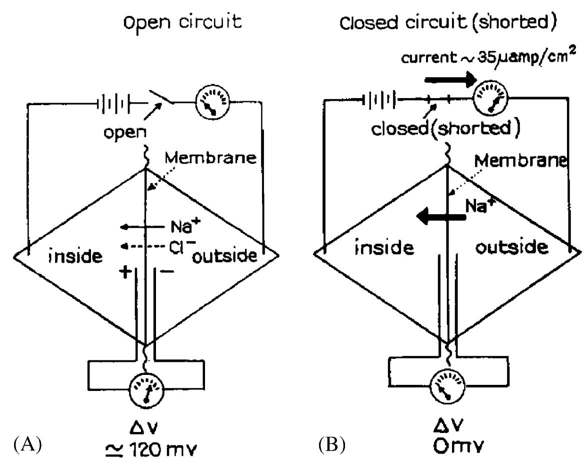

Fig. 3. Open- and closed-circuit recording in an Ussing chamber. In the open circuit a TEP is recorded that is due to the net difference between the voltages across apical and basal RPE membranes *plus* the voltage caused by any ionic current flowing extracellularly between the cells. In the short-circuit condition the TEP is held at zero by an applied current. When the apical and basal baths have identical ionic concentrations and the potential across the tissue is zero the electrochemical driving force upon sodium and potassium channels will be the same for both membranes and a negligibly small current should flow through such channels. However, the current resulting from active transport of ions will continue. This current is "backed off" by the applied current, and in RPE is the net efflux of Na^+ produced by the electrogenic Na–K–ATPase transporter. From Voûte and Ussing (1968). Reprinted with permission from the Journal of Cell Biology.

For work in mammalian RPE preparations at 37 °C, a modified Kreb's solution perfuses the apical and basal sides of the tissue. The chamber design allows for rapid changes of bath solution as well as the addition of agonists or inhibitors to investigate their effects on V_M (Miller and Steinberg, 1977a; Linsenmeier and Steinberg, 1983). Changes in the TEP can be caused by either alterations in V_{Basal} or V_{Apical}. A depolarisation of V_{Basal} causes the rise in the TEP observed in the light rise of the EOG (Gallemore and Steinberg, 1989b) and the c-wave of the ERG is associated with a hyperpolarisation of V_{Apical} (Oakley and Green, 1976).

The RPE can be modelled as an electrical circuit as in Fig. 4 (Miller and Farber, 1984). In the short-circuit state when the TEP = 0 mV, the resulting current is equal to i_{PUMP} and in bovine preparations this current is due to the active transport of Cl^- and HCO_3^- from the retinal to the choroidal surfaces and Na^+ in the opposite direction. Experiments in the open- and closed-circuit states using intracellular microelectrodes combined with radioactive flux measurements have shown how the changes in ionic fluxes accompany alterations in net fluid transport (Joseph and Miller, 1991; Kenyon et al., 1997; Miller and Edelman, 1990).

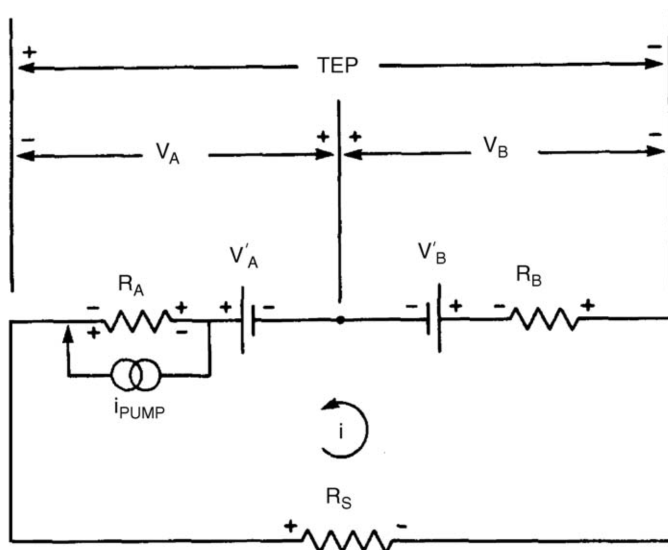

Fig. 4. The electrical circuit model of the RPE of Miller and Farber (1984) in which the apical and basal membranes are electrically coupled by a finite shunt resistance R_S that represents the paracellular resistance of the tight junctional complexes and edge tissue damage. The apical and basal membrane voltages (V_{Apical}) and (V_{Basal}) are represented by two batteries V'_{Apical} and V'_{Basal} in series with the apical and basal membrane resistances (R_{Apical}) and (R_{Basal}), respectively. At the apical membrane, an active transport current represented by i_{PUMP} is independent of membrane voltage. As the potential across the apical membrane is greater than that of the basal membrane (owing to the differences in ionic channels and conductances of each membrane) a shunt current (i) flowing from the apical to basal membrane, depolarises the apical membrane and hyperpolarises the basal membrane. Reprinted with permission of the Journal of General Physiology.

6.1.1. Fluid transport

Microelectrode studies of amphibian and mammalian RPE preparations have provided insights into the bulk movement of solutes and fluid. In light, the movement is from the subretinal space to the choroid (absorption) (Bialek and Miller, 1994; Edelman et al., 1994b; Hughes et al., 1984; Sellner, 1986; Tsuboi and Pederson, 1988) and in darkness the process reverses—from choroid to retina (secretion) (Edelman et al., 1994a, b). In mammalian, avian and amphibian species the apical membrane of the RPE contains the electroneutral Na–K–2Cl cotransporter, the Na–K–ATPase electrogenic pump and a large K^+ conductance (Miller and Edelman, 1990) that work together to control subretinal space and RPE cell-volume (Adorante, 1995; Adorante and Miller, 1990; Lin et al., 1992).

One method to determine the direction of ionic fluxes is to measure the net movement of trace amounts of radioactive isotopes from the apical to basal and basal to apical surface of the RPE mounted in an Ussing chamber in the open- and closed-circuit states. In the open circuit, the difference in unidirectional fluxes gives the net solute flux and the direction of fluid transport. In the closed-circuit state, the contribution to the ionic flux is that of the active transporters alone (Edelman et al., 1994a; Hughes et al., 1984; Miller and Farber, 1984; Rymer et al., 2001).

To record the minute volume changes over time, a rate of 4–6 μl/h/cm^2 (Hughes et al., 1984), a modified chamber was made from a water impermeable plastic in which each half chamber was sealed except for a small cannula that connected it to a vertical column of the bathing Kreb's solution. A capacitance probe was placed above each meniscus and as fluid flowed from either the apical or basal baths through the RPE the air gap above each meniscus changed and thus altered the capacitance as a direct function of fluid transport (Hughes et al., 1984; Miller et al., 1982; Peterson et al., 1997). For further details on these techniques see Gallemore et al. (1997) and Marmor (1990).

Application of ouabain to the apical but not basolateral membrane of frog RPE led to a rapid depolarisation of the apical membrane followed by a slower decrease in the TEP. This suggested that an apical Na–K–ATPase pump was present and potentially played a role in fluid regulation owing to its regulation of V_{Apical} (Miller et al., 1978; Miller and Steinberg, 1977a). However, later experiments showed that the Na–K–ATPase pump was not the main transporter involved with bulk fluid movement and in fact fluid transport was more sensitive to external HCO_3^- (Hughes et al., 1984). Furthermore, a role for intracellular second messengers such as cAMP was implicated as fluid absorption was reduced in its presence (Hughes et al., 1984, 1987, 1988). At the basolateral membrane a pH sensitive Cl^-–HCO_3^- antiporter was identified in frog that exchanged intracellular HCO_3^- for extracellular Cl^- (Lin and Miller, 1994). This system provided a means of coupling fluid transport to changes in pH_{in}. The TEP also provides a driving force for the passive transport of Na^+ ions via the paracellular pathway resulting in a net movement of NaCl

in light (Bialek and Miller, 1994; Tsuboi and Pederson, 1988). At the apical membrane, the increased HCO_3^- influx maintains V_{Apical} at a more hyperpolarised potential than V_{Basal} (Hughes et al., 1989).

In isolated frog eyecups, pH sensitive microelectrodes were used to record the changes in pH at different retinal depths in response to changes of light and dark. It was found that in light the pH of the subretinal space increased slightly (Borgula et al., 1989). This change could be attributed to either to a decrease in photoreceptor activity accompanying the decrease in dark current (Hagins et al., 1970) or a decrease in the activity of the apical $Na^+ \sim 2HCO_3^-$ cotransporter, leading to mild alkalisation of the subretinal space (Lin and Miller, 1991).

The direction of fluid transport is largely determined by alterations in subretinal $[K^+]_{out}$ (Oakley, 1983; Oakley and Green, 1976; Oakley and Steinberg, 1982). In darkness photoreceptors are active, and sodium flows into the outer limbs. It is expelled by a Na–K–ATPase exchange pump in the inner limb. Therefore a "dark current" circulates along the rod axis. In light, the outer limb sodium channels close, and the rod hyperpolarises. The pump continues for some time, depleting the subretinal space of potassium (Brown and Pinto, 1974; Korenbrot and Cone, 1972; Penn and Hagins, 1969; Sillman et al., 1969; Toyoda et al., 1969). This causes a hyperpolarisation of the apical RPE membrane that is one component of the c-wave of the ERG. The apical membrane potassium channel, the electroneutral Na–K–2Cl cotransporter and the electrogenic $Na^+ \sim HCO_3^-$ cotransporter respond to changes in subretinal $[K^+]_{out}$. When the dark current is present and the subretinal $[K^+]_{out}$ is $\sim$5 mM then V_{Apical} is largely maintained by the potassium concentration gradient across it, and under these conditions, the $Na^+ \sim HCO_3^-$ cotransporter is active passing sodium and bicarbonate into the cell down the favourable electrochemical gradient: the sodium is then pumped out again, in exchange for potassium (there is also an apical $Na^+–H^+$ that also regulates pH_{in} (Lin et al., 1992; Zadunaisky et al., 1989). The net result is a mild alkalinisation of the RPE cytosol. This elevated pH_{in} stimulates the *basolateral* $Cl^-–HCO_3^-$ exchanger which causes an enhanced basal flux of Cl^- into the RPE. Chloride ions then exit through the apical membrane (la Cour, 1991a, b). Light onset results in decreased potassium in the subretinal space and therefore the apical surface of the RPE hyperpolarises. The hyperpolarisation reduces the entry of negative ions like HCO_3^- into the RPE, (Oakley, 1977) resulting in a decrease of pH_{in} and a decrease in the exchange rate of the basolateral $Cl^-–HCO_3^-$ (Tsuboi and Pederson, 1988; Bialek and Miller, 1994). This explains how changes in retinal illumination alter the direction of fluid transport (Edelman et al., 1994a, b; Lin and Miller, 1991, 1994). See Fig. 5 which represents work mainly on amphibian RPE preparations. For further reviews of RPE fluid transport and the equivalent circuit derivations, see Gallemore et al. (1997) and Steinberg et al. (1983, 1985).

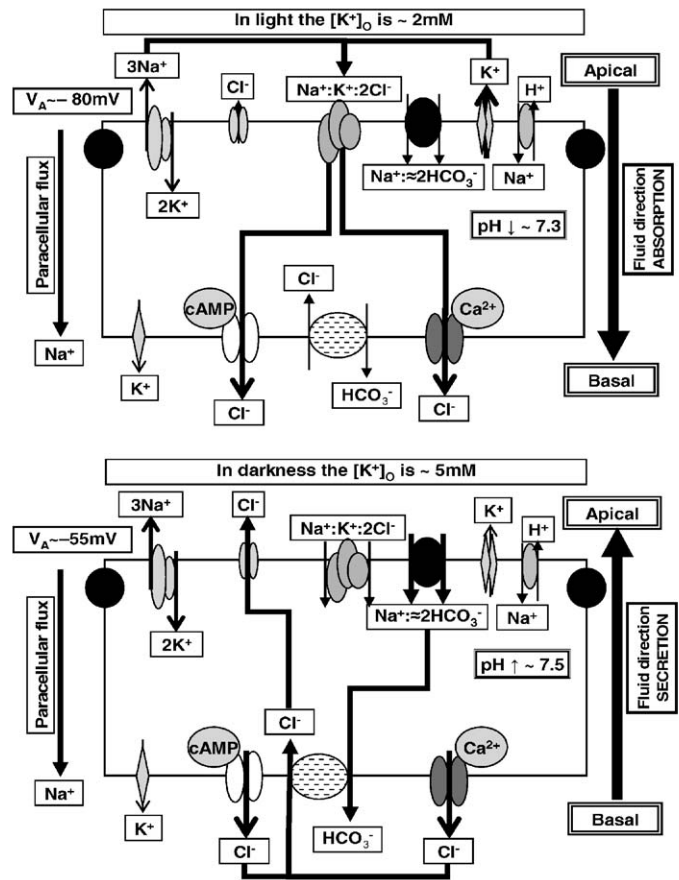

Fig. 5. Schematic representations of the ionic fluxes in the RPE during light (top) and in dark (bottom). In light, there is steady state absorption of fluid and salts from in the retina to choroid maintained by the apical active Na–K–ATPase pump that extrudes Na^+ against its electrochemical gradient. Arrows approximate the relative contribution of each channel to ionic fluxes. The apical Na–K–2Cl cotransporter uses the energy from the Na^+ gradient to return Cl^- into the cell against its electrochemical gradient. At the basolateral membrane, Cl^- leaves via chloride channels (two types are shown in the diagram). *In light*, the intracellular pH is $\approx$7.3 because the apical membrane $Na^+–H^+$ antiporter exchanges sodium for hydrogen ions, and the influx of HCO_3^- decreases as the apical membrane hyperpolarises creating an unfavourable electrochemical gradient for HCO_3^-. *In darkness*, when the rod dark current is restored there is a rise in subretinal $[K^+]$ and the apical membrane depolarises. This apical depolarisation then increases the influx of sodium and bicarbonate by the $Na^+ \sim 2HCO_3^-$ cotransporter and as a result the cell interior alkalises to pH $\approx$7.5. The elevated intracellular $[HCO_3^-]$ then activates the basolateral $Cl^-–HCO_3^-$ antiporter that reverses the direction of net fluid movement from absorption to secretion by returning Cl^- ions back to the subretinal space. Figure based upon those of Hartzell and Qu (2003), Quinn and Miller (1992).

Whilst the Ussing chamber and intracellular recordings provided valuable insights into the mechanisms involved in regulation of cell pH, the volume of the subretinal space and the principal transporters involved in these activities, the underlying kinetics and properties of these ionic channels required a different technique that enabled recordings to be made from single cells and isolated populations of ionic channels in which the intra and extracellular environment can be controlled.

6.2. Patch clamp

Hodgkin's and Huxley's work on the squid giant axon enabled them to record membrane voltage and current, and also to control both the internal and external ionic composition. This was not possible with *small* cells until the development of the patch clamp technique (Hamill et al., 1981; Hamill and Sakmann, 1981; Neher and Sakmann, 1976; Neher et al., 1978; Sigworth and Neher, 1980). A glass micropipette with a tip diameter of 0.5–1 μm is heated (fire polished) in a microforge until its tip is smooth. It is filled with a solution that mimics the intracellular ionic composition and then advanced to the cell membrane, which adheres to the glass so that the resistance between the interior of the pipette and the extracellular fluid rises to $>100\,M\Omega$. If suction is applied to the pipette, the resistance may rise to 10^9–$10^{11}\,\Omega$. This is known as the *cell-attached* configuration. A second (reference or bath) electrode is placed in the bath surrounding the cell: usually it is a Ag/AgCl pellet connected via an agar–salt bridge.

If the cell membrane in the microelectrode tip contains channels or other ionic transporters the resistance of the patch is less than between microelectrode and the bath. The total membrane resistance of the rest of the cell is very much lower, and therefore the voltage between microelectrode and bath approximates to the membrane voltage minus a liquid junction potential between microelectrode tip and bath fluid (due to the different ionic compositions) that must be determined experimentally or calculated (Barry and Lynch, 1991; Neher, 1992; Ng and Barry, 1994). Any small current flowing causes a relatively large voltage change inside the patch electrode. Thus the current of single channels can be monitored.

When V_M is held or clamped at a range of hyperpolarising and depolarising voltages for 50–250 ms the membrane current changes and stabilises, and after 1–2 s another "holding potential" can be applied. Software does this automatically and provides a plot of the *I–V* curve. The slope indicates the instantaneous conductance at each of the voltage steps. An ion channel giving a simple linear current/voltage relationship over the entire range of membrane voltage has an Ohmic *I–V* curve. An example of such an ionic channel is the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989; Rommens et al., 1989) located at the apical and basal membranes of foetal RPE (Blaug et al., 2003). Rectifying channels have non-Ohmic *I–V* curves. An inward rectifying channel is one in which a hyperpolarising voltage produces a greater inward current than the outward current produced by an equivalent depolarising step. Therefore, in such an *I–V* curve the slope is greater at membrane voltages more negative than the resting membrane voltage. An example is the K^+_{Vir} channel found in the apical membranes of the RPE (Hughes and Takahira, 1996). The outward rectifying currents have the reverse characteristic. The conductance increases for depolarising membrane steps more than it decreases for hyperpolarising voltages steps. An example is the K^+ outward rectifier identified in human (Hughes et al., 1995) and bovine RPE (Takahira and Hughes, 1997).[3]

7. Origins of the light rise

The slow rise in the TEP in animal preparations is related to the light rise in the human light-EOG. It is caused by a depolarisation of the basal membrane of the RPE, so the net difference between basal and apical membrane voltages increases. This slow rise must be produced by a "second messenger" within the cytosol of the RPE. Therefore, for the light rise the entire sequence must be that the retina liberates a chemical (referred to as the "light substance") that, binding to a receptor in the apical membrane of the RPE, liberates an internal "second messenger". The second messenger causes the increase in basal Cl^- conductance. The light-EOG is shown in Fig. 1.

[3]Once a GΩ seal is formed in the *cell-attached* mode there are several possible manipulations of the cell membrane in which to explore the ionic channels and their properties (Hamill and Sakmann, 1981; Hamill et al., 1981). In whole cell recordings suction is applied to the microelectrode to break the patch, which then makes a low-resistance pathway to the cell interior. Then, in small cells the total average response of all the ionic channels in the cell membrane can be recorded. However, cytosolic signalling molecules are diluted by mixing with the solution in the micropipette, and the pipette solution cannot be easily modified (Lapointe and Szabo, 1987; Soejima and Noma, 1984; Tang et al., 1990).

The *perforated whole cell* configuration better maintains intracellular contents. The micropipette contains nystatin (Horn and Marty, 1988) or amphotericin B (Rae et al., 1991). These substances form small pores within the cell membrane through which large molecules and organelles cannot pass. This configuration that has been widely used to examine the currents in amphibian, mammalian and cultured RPE cells (Hartzell and Qu, 2003; Hughes and Segawa, 1993; Hughes and Steinberg, 1990; Hughes and Takahira, 1998; Mergler et al., 1998; Rosenthal and Strauss, 2002; Sheu and Wu, 2003; Strauss et al., 1993, 1997, 1999, 2000; Strauss and Wienrich, 1993, 1994; Takahira and Hughes, 1997; Ueda and Steinberg, 1994, 1995; Valtink et al., 1999; Wen et al., 1993, 1994; Weng et al., 2002; Wills et al., 2000). Manipulation of the pipette and bath solutions can be used to isolate specific ionic currents in the whole cell. For example, addition of $BaCl_2$ to the bath solution will intensify Ca^{2+} currents and the addition of CsCl and tetraethylammonium inhibits K^+ currents (Ueda and Steinberg, 1993, 1995).

Alternative configurations can be made once the seal has been established. In the *inside-out* configuration after the patch has formed, the pipette is withdrawn, so that the membrane is torn from the cell. The bath fluid becomes equivalent to the intracellular environment and can be modified (Hamill and Sakmann, 1981; Hamill et al., 1981). The small excised patch contains only a few channels and when these open and close large proportionate changes in total membrane conductance occur. Therefore, the characteristics of the ionic channels can be investigated fully.

Once the microelectrode has made contact with the surface of the cell, suction applied slowly allows the cell membrane to balloon into the tip. Eventually the tip becomes sealed in such a way that the inside of the cell membrane faces the pipette solution. The pipette is then withdrawn, and separated from the rest of the cell membrane. The outer membrane surface now faces the bath forming the *outside-out* configuration. Direct activation of ionic channels by agonists added to the bath is made easier but this configuration is difficult to achieve unless the cell is attached firmly so that enough force can be applied to remove the patch from the cell membrane (Hamill and Sakmann, 1981; Hamill et al., 1981).

The most notable contributions are by Roy Steinberg and Sheldon Miller and their collaborators (Bialek et al., 1995; Gallemore et al., 1988; Gallemore and Steinberg, 1989a, b, 1993; Griff and Steinberg, 1982, 1984; Joseph and Miller, 1991; Linsenmeier and Steinberg, 1982, 1983, 1984; Oakley and Steinberg, 1982; Valeton and van Norren, 1982).

7.1. What cells initiate the response?

Griff and Steinberg (1982), Linsenmeier and Steinberg (1982), Valeton and van Norren (1982) used a combination of intra-retinal microelectrodes, combined with reference electrodes sited in the subretinal space or retro-ocularly, to prove that the light rise originated in the RPE (see Fig. 6). Previously it had been supposed that much of the voltage change could develop in the retina. The mechanism of production was investigated by measuring the spectral sensitivity of the process. There was a simple relationship to light intensity, and blue green light being most effective, showing that the light that was being absorbed by the rods. Similar results in man had been described by Arden and Kelsey (1962b). But how then could the rods influence the RPE? Steinberg noted that the change in the RPE was dependent on stimulus area (Linsenmeier and Steinberg, 1982). The obvious explanation was that some substance generated by light, moved from the rods to the RPE: the concentration depended upon the light intensity, and also upon whether the substance could diffuse away into unilluminated portions of the subretinal space. Such experiments did not prove that the light rise substance was generated in photoreceptors: the light rise might be produced as a post-synaptic result of rod activity, as was proposed by Gouras and Gunkel (1963), who found that in central retinal vein occlusion, the light-EOG was reduced. However, Gallemore et al. (1988) showed that when post-receptoral activity was blocked the light rise was unaffected thereby demonstrating that the light rise substance, however it was generated, was released by the rods. The absence of the light rise in patients with early retinal dystrophies also provides evidence that the light rise substance is generated in rods (Arden, 1962).

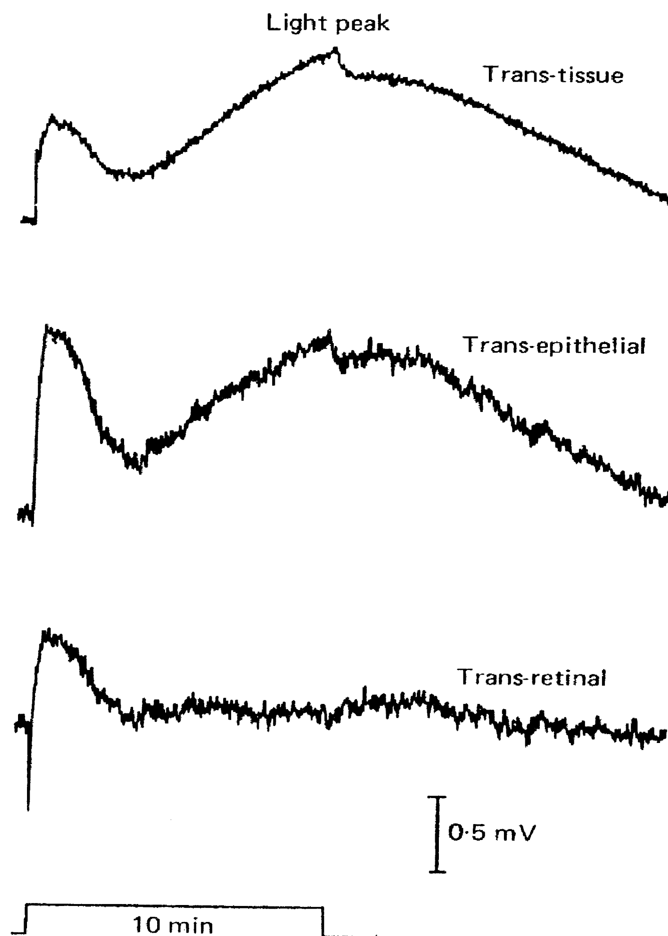

Fig. 6. Intracellular microelectrode recordings from the RPE in a retina–RPE preparation of Gecko. Voltages across the apical and basal faces of the RPE are recorded separately by extracellular electrodes placed in the subretinal space or in choroidal side of the RPE. These electrodes also recorded the TEP. The light rise potential develops across the RPE (middle trace) with no contribution to the potential by the retina (lower trace). From Griff and Steinberg (1982). Reprinted with permission of the Journal of Physiology.

7.2. RPE involvement and the basolateral membrane

In order to quantify the contribution of the apical and basal membranes to the EOG as well as the role of the neural retina in the generation of the light rise, preparations of intact RPE–retina were mounted in modified Ussing chambers. The resistance across the tissue fell during the light peak. By introducing a microelectrode into the RPE it was possible to record V_{Basal} and V_{Apical} differentially by referencing the intracellular electrode to the basal and apical bath electrodes, respectively. Griff and Steinberg (1982) were able to show that during the light peak, net membrane voltage decreased, but the basal membrane depolarised more than the apical membrane, increasing the TEP. However, these observations did not prove which membrane caused the light rise. Griff and Steinberg (1982), Linsenmeier and Steinberg (1983) passed current pulses between apical and basal electrodes while monitoring the membrane voltage changes caused across each membrane ($\Delta V_{\mathrm{Apical}}$ and $\Delta V_{\mathrm{Basal}}$). Some of the injected current passes via the paracellular pathway in parallel to the membranes and so the absolute values of the resistance of apical and basal membranes could not be determined. Instead, the ratio of the resistances (the a-value) was obtained (Eq. (5)).

$$a = \Delta V_{\mathrm{Apical}}/\Delta V_{\mathrm{Basal}} = R_{\mathrm{Apical}}/R_{\mathrm{Basal}} \quad (5)$$

when the a-value is taken together with the changes in total tissue resistance then it is possible to determine at which membrane the greatest change in resistance occurs. In lizard (Griff and Steinberg, 1982), cat (Linsenmeier and Steinberg, 1983) and chick (Gallemore et al., 1988; Gallemore and Steinberg, 1989b, 1991), V_{Basal} depolarises

in response to light with a rise in conductance, but the voltage change across the apical membrane was not accompanied by a change of conductance. Today it is technically possible to measure the paracellular resistance directly, without the contribution of ''edge effects'' (Gitter et al., 1997).

7.3. Chloride channels

The depolarisation of the basal membrane could be achieved either by a negative ion exiting the cell or a positive ion entering the cell. Evidence that the light rise was mediated by an increase in basolateral Cl^- conductance was shown when four Cl^- channel blockers all abolished the light rise in chick (Gallemore and Steinberg, 1989b). Furthermore, Cl^- sensitive microelectrodes inserted into chick RPE showed a decrease in the intracellular Cl^- activity that matched the light rise (Gallemore and Steinberg, 1993). Therefore, negative Cl^- ions leaving through the basolateral membrane must cause the light rise. From this it follows that the light rise substance must initiate the release of some second messenger within the RPE that in its turn modulates basolateral membrane Cl^- conductance.

However, the identity of the Cl^- channels responsible for the EOG and FOs has not been fully resolved, but with the advent of new compounds that are able to selectively block chloride channels, this should soon be accomplished. It should be noted that different species might have different types of Cl^- channel, which may not all respond in the same way to the second messengers. Another issue is the role of calcium in regulating the basolateral Cl^- currents that cause the light rise Hofmann and Niemeyer (1985) showed that the light rise was reduced by an increase of $[Ca^{2+}]_{out}$ and so Ca^{2+} is presumed to play some role in initiating the light rise. CFTR is not directly affected by Ca^{2+} and as the amplitude of the light rise is normal in cystic fibrosis (CF) this Cl^- channel is not thought to be directly involved in the light rise (Miller et al., 1992; Lara et al., 2003). It is likely that the Cl^- channel responsible for the light rise is gated by Ca^{2+} and current evidence suggests that bestrophin is the channel responsible (see below).

8. Signalling pathways

The study of fluid transport in Ussing chambers along with microelectrode recordings and patch clamp experiments has yielded a large understanding of the ionic currents found within the RPE (Gallemore et al., 1997). Regulation of the ionic channels and gating by second messengers is fundamental to the generation of the light-EOG and FOs and so we will briefly outline the key signalling pathways and messengers that are involved in ionic channel gating. For a detailed review of second messenger signalling pathways in the RPE see Nash and Osborne (1996).

8.1. G-proteins

One way in which a cell communicates with the external environment is via special membrane-spanning proteins that combine with particular chemicals (ligands) in the extracellular fluid. The communication is via an enzyme cascade that amplifies the signal. The intracellular terminals are associated with specialised *G*-proteins, (often distinguished by a subscript, e.g. G_s or G_q) which dissociate from the receptor when it binds to the extracellular signal. The receptor binds extracellularly, often to a single ligand which can be an ion, organic odorant, amine, peptide, protein, lipid or nucleotide. In the olfactory receptors in the nose, the receptor binds to odoriferous substances (Breer, 2003) and in the photoreceptor, rhodopsin, the receptor molecule, absorbs a photon of light. The first suggestion that a conformational change in a protein could lead to a sequence of biochemical changes in the eye was due to Wald (1968), who postulated that the cis–trans isomerisation of rhodopsin by a photon could initiate phototransduction. The binding of the ligand to the receptor initiates the first enzymic step, the activation of *G*-proteins (for review see Kristiansen (2004)) which consist of heterotrimeric isomers (proenzymes) ($G_{\alpha\beta\gamma}$) (Cabrera-Vera et al., 2003; Gilman, 1987; Pierce et al., 2002) that dissociate into G_α and $G_{\beta\gamma}$ complexes. The dissociated fragments are active enzymes, which are capable of acting synergistically or as antagonists to rapidly alter the activity of ionic channels and intracellular signalling molecules. For example, G_s is coupled to the β-adrenergic receptor which when stimulated causes its dissociation into an active enzyme, whose function is to transform another proenzyme, membrane bound adenylate cyclase (AC) (Rodbell et al., 1971) into a fully active enzyme that forms cAMP utilising ATP as a substrate (Friedman et al.,1987). Generation of cAMP then activates protein kinase-A (PKA) that is able to phosphorylate proteins and ionic channels and thereby modulate their function (Anderson et al., 1991c). Another example is photoexcited rhodopsin which complexes with the *G*-protein (transducin) that then activates membrane bound phosphodiesterases. The latter catalyses the hydrolysis of cyclic guanosine monophosphate (cGMP) to GMP which closes down outer segment cation channels (Fung et al., 1981; Fung and Stryer, 1980).

A different but equally important intracellular transduction pathway is provided by G_q which activates phospholipase C-β (Smrcka et al., 1991) through hydrolysis of the plasma membrane phospholipid phosphatidylinositol-4,5-bisphosphate, (Mitchell, 1982a, b) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) which then act as second messengers to raise $[Ca^{2+}]_{in}$ which then modulates the activity of ionic channels either directly or through the activation of protein kinase-C (PKC). The conductance of some ionic channels changes when they are phosphorylated by PKC (Strauss et al., 1997). IP_3 binds to a receptor on the endoplasmic reticulum (ER) to release intracellular Ca^{2+} (see below). DAG is also involved in the

release of the polyunsaturated fatty acid arachidonic acid from the plasma membrane which is metabolised into second messengers (Piomelli and Greengard, 1990; Shimizu and Wolfe, 1990; Sigal, 1991).

8.2. Protein tyrosine kinases

One additional means of ionic channel control is through protein tyrosine kinases (PTKs) that are membrane-bound proteins that act as enzymes by directly phosphorylating tyrosine residues in intracellular target proteins after extracellular activation by growth factors such as epidermal growth factor, insulin or pigment-derived growth factor (Glenney, 1992; Schlessinger and Ullrich, 1992). Integration of PTKs and *G*-protein signalling pathways provides an alternative means by which *G*-protein activation and signalling pathways can interact with PTKs to modulate cell physiology (Waters et al., 2004). Of interest in this review are the non-receptor tyrosine kinases that belong to the *src* protein family and lack an extracellular binding domain but retain an intracellular tyrosine kinase catalytic domain. These PTKs also play a role in the regulation of Ca^{2+} channels and homeostasis and will be discussed later (Strauss et al., 1997, 2000; van der Heyden et al., 2005).

9. Ionic channels of the RPE involved in the EOG

9.1. The calcium activated chloride channel

A rise in intracellular calcium $[Ca^{2+}]_{in}$ is believed to generate the light rise by gating open a basolateral calcium-activated chloride channel (CaCC). It should be noted that evidence for Ca^{2+} being the intracellular second messenger is indirect, and based upon normal light rises in CF with abnormal FOs (Lara et al., 2003; Miller et al., 1992). In the artificially perfused cat eye a reduction in the light rise occurs when extracellular $[Ca^{2+}]$ is increased, although the FOs and c-wave are unchanged (Hofmann and Niemeyer, 1985). The abnormal FOs in CF implicates cAMP and CFTR in the generation of this response. Therefore, the presence of a normal light rise in CF suggests the ionic mechanisms are different for the FOs and light rise (Blaug et al., 2003). Furthermore, the FOs are normal in Best's with a reduction in the light rise which further suggests that the light rise and FOs have a different underlying mechanism with presumably different basolateral ionic channels responsible (Weleber, 1989). (See below for the differential effect of abnormalities of bestrophin on the light rise and FOs for additional evidence).

CaCCs were first identified in *Xenopus* oocytes where a rise in $[Ca^{2+}]_{in}$ following fertilisation led to a depolarisation of the oocytes that prevented further entry of sperm (Barish, 1983; Miledi, 1982). CaCC is crucial for fluid regulation in the RPE (Peterson et al., 1997; Rymer et al., 2001) as it is in the genesis of many other secretions such as saliva (Melvin et al., 2005) and tears (Mircheff, 1989). A partially inhibitable CaCC current by 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) was first identified in neo-natal rat RPE (Ueda and Steinberg, 1994). However, these currents were short-lived and not always recordable indicating that they were probably dependent upon a compound that was lost during the whole-cell recordings (Botchkin and Matthews, 1993).

In cultures of normal rat RPE, the CaCC currents were stimulated by release of Ca^{2+} from the ER stores via IP_3. To avoid depletion of calcium stores in rat RPE, PTKs initiate an influx of Ca^{2+} from the extracellular space via a voltage gated L-type Ca^{2+} channel which is described below (Strauss et al., 1997, 1999).[4] CaCC currents have been reported in *Xenopus* but display different activation and kinetics (Hartzell and Qu, 2003; Strauss et al., 1999). CaCCs are also sensitive to pH_{in} and may help to buffer extracellular acidification by HCO_3^- conductance (Zhang et al., 1992).

Bovine and human foetal RPE monolayers mounted in Ussing chambers all show an increased basolateral Cl^- conductance following elevation of $[Ca^{2+}]_{in}$ provoked by adrenergic agonists, epinephrine and isoproteneroI (Quinn et al., 2001; Rymer et al., 2001), synthetic or ATP purinergic agonists (Maminishkis et al., 2002; Peterson et al., 1997), and the non-steroidal anti-inflammatory drug, niflumic acid (but not flufenamic acid) (Bialek et al., 1996). Whether Ca^{2+} binds directly to the protein or acts via calmodulin-dependent protein kinase remains to be determined. All these agents stimulate a DIDS-sensitive increase in basolateral Cl^- conductance. The Cl^- channel blockers available show some tissue specificity, with glibenclamide and flufenamic acid showing no inhibition of CaCC and CFTR in rat cerebral arteries (Doughty et al., 1998). Generally, the permeability sequence of CaCC obtained from smooth muscle, endothelial cells, lacrimal glands and *Xenopus* oocytes is $SCN^- > NO_3^- > I^- > Br^- > Cl^- > F^-$ (Cornejo-Perez and Arreola, 2004; Evans and Marty, 1986; Large and

[4] In the RCS rat (Bourne et al., 1938; Bourne and Gruneberg, 1939) where the RPE fails to phagocytose rod outer limbs and a retinal degeneration results, it has been shown that this calcium channel is abnormal (Edwards and Szamier, 1977; Gal et al., 2000). Its regulation is altered allowing a greater inward Ca^{2+} current (Strauss and Wienrich, 1993). This may be an important cause of the inability to phagocytose rod outer segments (Hall et al., 1991, 2002; Heth et al., 1995; Mergler et al., 1998; Rodriguez de Turco et al., 1992; Salceda, 1992). The chloride channel blockers, niflumic and flufenamic acid, and DIDS and SITS all modify the kinetics of voltage gated K^+ channels in myocytes (Wang et al., 1997). Mefenamic and flufenamic acids also activate a K^+ conductance in smooth muscle cells of the gut (Farrugia et al., 1993). Flufenamic and niflumic acids also activate large-conductance Ca^{2+}-gated K^+ channels (Gribkoff et al., 1996; Ottolia and Toro, 1994) and these drugs blockers also inhibit nonselective cation channels (Chen et al., 1993; Gogelein et al., 1990). In mast cells separate membrane Ca^{2+} and Cl^- currents were inhibited by DIDS whilst only the Cl^- current was inhibited with niflumic acid (Reinsprecht et al., 1995). The interactions of the various Cl^- channel blockers with other ionic channels has made interpretations of Cl^- channel function difficult and often high doses of these drugs are required to have an effect (Frings et al., 2000; Hartzell et al., 2005a; Jentsch et al., 2002; Qu and Hartzell, 2000).

Wang, 1996; Nilius et al., 1997; Qu and Hartzell, 2000). This is the same as the sequence for bestrophin (Qu et al., 2004) which also generates calcium-dependent Cl^- currents (Qu et al., 2003; Sun et al., 2002; Tsunenari et al., 2003).

9.2. Bestrophin—a CaCC?

Best's vitelliform macular dystrophy is an inherited autosomal dominant disease with a variable age of onset from childhood to adulthood (Renner et al., 2005) and is characterised by a central vitelliform (poached egg) lesion at the macula. The retina over the lesion becomes damaged, leading to loss of visual acuity. However, the underlying fundamental process is found throughout the entire retina because the light rise of the EOG is absent (Barricks, 1977; Cross and Bard, 1974; Deutman, 1969; Francois et al., 1967; Renner et al., 2005; Thorburn and Nordstrom, 1978).[5]

The gene responsible for Best's (VMD2) has been identified (Forsman et al., 1992; Marchant et al., 2001; Marquardt et al., 1998; Petrukhin et al., 1998; Stöhr et al., 2002; Stone et al., 1992; White et al., 2000; Yoder et al., 1988). It codes for a protein, designated bestrophin, which is specifically expressed in the basal surface of the human, porcine and macaque RPE (Marmorstein et al., 2000). Cultured foetal human RPE cells express bestrophin when grown in specifically defined medium (Hu and Bok, 2001) while the human ARPE-19 (Dunn et al., 1996) and D407 (Davis et al., 1995) and rat (RPE-J) (Nabi et al., 1993) RPE cell lines are positive at the messenger level for bestrophin. The protein was not detected using western blots in these cell lines (Marmorstein et al., 2000).

Bestrophin was predicted to form an ionic exchanger based upon the amino acid sequence (Gomez et al., 2001). Transfection of one of several isoforms of human bestrophin (hBest1) into cell lines demonstrated anionic currents with mutant bestrophin inhibiting wild-type bestrophin currents that were activated by $[Ca^{2+}]_{in}$ (Qu et al., 2003, 2004; Sun et al., 2002; Tsunenari et al., 2003). Hartzell et al. (2005a, b) demonstrated that site-directed mutagenesis of bestrophin pores altered their SCN^- conductance. Expression of mouse bestrophin (mBest2) into mammalian cell lines also demonstrated that the product of mBest2 formed a Cl^- channel whose permeability and anion selectivity could be altered by mutations that strongly suggested that bestrophin was a Cl^- channel (Pusch, 2004; Qu and Hartzell, 2004).

[5]There are a few caveats to be made: Whilst similar lesions occur in 'pseudovitelliform dystrophy' there is no significant reduction in the light-EOG (Epstein and Rabb, 1980; Kingham and Lochen, 1977; Marmor, 1979; Sabates et al., 1982; Theischen et al., 1997). Furthermore, some individuals with VMD2 and vitelliform lesions have normal light-EOGs (Pollack et al., 2005). Histopathological studies in Best's reveal abnormalities of the RPE, neovascularisation, photoreceptor degeneration and accumulation of lipofuscin and melanolipofuscin but these do not pinpoint the fundamental cause of the disease (Frangieh et al., 1982; O'Gorman et al., 1988; Weingeist et al., 1982).

Two human bestrophin homologues investigated (hBest1 and hBest2) have different permeability sequences. They show a high permeability for NO_3^- that suggests bestrophin is also permeable to bicarbonate ions (Sun et al., 2002). *I–V* curves for four human bestrophin proteins have different characteristics with hBest2 and hBest4 being almost linear and hBest3 shows strong inward rectification and hBest1 has moderate outward rectification (Sun et al., 2002; Tsunenari et al., 2003).

The way bestrophin might regulate the light rise is not completely determined (some bestrophin mutations can produce phenotypes that are different to classical Best's disease). In addition to the calcium activation described above, there are also phosphorylation sites that may regulate gating (Marmorstein et al., 2002). In one study mutant versions of bestrophin were expressed in basal membrane of RPE following subretinal injections of an adenovirus vector into rats. The authors found that while wild type increased the light rise, the constructs from Best's (W93C and R218C) selectively reduced (and changed the time course of) the light rise, and could thus serve as a model for Best's disease. There were no significant effects on the FOs or ERG components, but detailed analysis of the sensitivity to light revealed some differences between the rat model and human patients (Marmorstein et al., 2004). The likely candidate for the light rise Cl^- channel is bestrophin. Two bestrophin genes cloned into *Xenopus laevis* oocytes express Ca^{2+}-activated Cl^- currents (Qu et al., 2003, 2004; Qu and Hartzell, 2004).

The molecular identity of this "ionic channel" is unknown despite the fact that the basic voltage dependence, anion selectivity and kinetics of this family of channels has been described (Eggermont, 2004; Qu et al., 2003). Recent evidence suggests that bestrophin regulates Ca^{2+} entry into the RPE via an L-type Ca^{2+} channel (Rosenthal et al., 2005; Strauss and Rosenthal, 2005) and this mediated influx of Ca^{2+} may act directly upon bestrophin or a separate CaCC to increase basolateral Cl^- conductance. There are reports of normal light rises in Best's which further complicate this issue (Lorenz and Preising, 2005; Pollack et al., 2005). This issue will finally be resolved with the demonstration that the light rise is absent in a knock-out animal model of Best's. However, the animal studies to date support the model in which bestrophin is the basolateral Cl^- channel responsible for the light rise (Hartzell et al., 2005b; Marmorstein et al., 2000, 2004; Pinckers et al., 1996; Ponjavic et al., 1999; Pusch, 2004; Qu et al., 2003, 2004). Recent evidence suggests that bestrophin also plays a role in volume regulation with decreasing current in response to hyperosmotic solution (Fischmeister and Hartzell, 2005).

For further reviews on the molecular identity and electrophysiology of Cl^- channels, see Begenisich and Melvin (1998), Fuller and Benos (2000), Hartzell et al. (2005a, b), Jentsch et al. (2002), Nilius and Droogmans (2003), Pusch (2004).

10. Calcium channels of the RPE

Calcium plays an important role in intracellular signalling sequences (second messengers). In cultures of human RPE a variety of peptides, growth factors, amino acids and agonists have been shown to elevate $[Ca^{2+}]_{in}$ via IP_3 generation and release of intracellular Ca^{2+} stores (Ammar et al., 1998; Feldman and Randolph, 1993; Fragoso and Lopez-Colome, 1999; Kuriyama et al., 1991, 1992; Quinn et al., 2001). In addition, Ca^{2+} is required for binding and ingestion of outer rod segments in cultured rat RPE cells and together with PKC plays a role in inhibiting phagocytosis (Hall et al., 1991, 2001, 2002). Thus Ca^{2+} homeostasis is vital for the RPE and the regulation of free cytosolic $[Ca^{2+}]_{in}$ is the responsibility of channels, pumps and transporters located on the plasma membrane and intracellular organelles. Resting cytosolic free $[Ca^{2+}]_{in}$ in human cell cultures is estimated to be ~70–200 nM (Feldman and Randolph, 1993; Feldman et al., 1991; Kuriyama et al., 1991, 1992) which is significantly lower than the millimolar extracellular concentration. However, the total $[Ca^{2+}]_{in}$ in the RPE cell is higher due to storage in both the ER and in melanosomes (Hess, 1975; Salceda and Riesgo-Escovar, 1990; Salceda and Sànchez-Chàvez, 2000). Ion selective microelectrode recordings in isolated cat RPE–retina show that the $[Ca^{2+}]_{out}$ in the subretinal space decreases during illumination with the restoration depending upon the RPE (Gallemore et al., 1994; Livsey et al., 1990). Concurrently, the volume of the subretinal space increases and during darkness must be reduced, a process critically dependent upon $[Ca^{2+}]_{in}$ (Li et al., 1994a, b). The sequence appears to be that the rise in the volume of the subretinal space deforms the RPE's membrane which leads to "Ca^{2+} signalling" via gap-junctions in normal rat RPE cell cultures. It is of interest that in the Royal College of Surgeon's (RCS) rat RPE cultures (Himpens et al., 1999; Stalmans and Himpens, 1997) such signalling is reduced. Elevation of $[Ca^{2+}]_{in}$ stimulates fluid transport from the subretinal space by increasing basolateral Cl^- transport (Maminishkis et al., 2002; Mitchell, 2001; Peterson et al., 1997; Rymer et al., 2001). For further reviews on Ca^{2+} channels in the RPE, see Rosenthal and Strauss (2002).

10.1. Na^+/Ca^{2+}-exchanger and Ca^{2+}-ATPase pump

Efflux of Ca^{2+} from the cytosol is in part accomplished by utilising the favourable high extracellular Na^+ concentration maintained by the Na–K–ATPase exchanger. The sodium–calcium exchanger (NCX) was first identified in heart (Reuter and Seitz, 1968) and the squid axon (Baker et al., 1969). Three members of this family have been identified. NCX1 is expressed ubiquitously and transports 3 Na^+ for 1 Ca^{2+} while NCX2 and NCX3 are limited to brain and skeletal muscle. An NCX was identified in the apical membrane fraction of bovine and fish RPE cells and is inhibited by bepridil (Fijisawa et al., 1993). One proposed function of the NCX is the regulation of Ca^{2+} concentration in the subretinal space following light onset. In man, cardiac NCX1 is present in both retina and RPE although localisation to the apical or basal membranes in the RPE was not possible (Loeffler and Mangini, 1998; Mangini et al., 1997). This exchanger is extremely fast and efficient and in heart muscle is responsible for the ending of contraction by reducing cytosolic calcium. In the RPE it could cause equally important and rapid changes, particularly since the RPE expresses voltage-dependent Ca^{2+} channels, but to date there have been no further studies investigating the its role in the RPE despite its role in Ca^{2+} homeostasis.

Another mechanism that moves calcium across the plasma membrane is the (Ca^{2+}–Mg^{2+})-ATPase pump (PMCA) (Kennedy and Mangini, 1996). PMCA hydrolyses ATP to actively transport Ca^{2+} against the concentration gradient and thus, removes Ca^{2+} from the RPE's cytosol and thereby helps to maintain Ca^{2+} homeostasis in the cytosol. It is not known whether PMCA in RPE is localised to apical or basal surfaces of the RPE (Kennedy and Mangini, 1996). There may be several isoforms of this channel (Johnson et al., 1995). For further reviews, see Philipson and Nicoll (1993) and Quednau et al. (2004).

10.2. Voltage-gated Ca^{2+} channels (L-type)

Whilst NCX1 and the PMCA provide a means of Ca^{2+} exit from the RPE cell a means of Ca^{2+} influx is also required. Voltage-dependent L-type Ca^{2+} currents with a very long time constant of de-activation (L = "long") were first observed in the heart (Orkand and Niedergerke, 1964). The inactivation maintains long depolarising responses and provides a means for sustained Ca^{2+} entry into the RPE down the favourable electrochemical gradient. L-type Ca^{2+} channels are blocked by 1,4-dihydropyrimidines such as nifedipine, phenylalkylamines and benzothiazepines. They are formed by five protein subunits (α_1, α_2, β, δ and γ) with the α_1 subunit forming the pore and defining the type (Knaus et al., 1990). See Catterall (2000) and Perez-Reyes and Schneider (1995) for further details.

Such voltage-gated Ca^{2+} channels were first recorded in fresh and cultured RPE cells from normal and dystrophic rats (< 17 days post natal) (Ueda and Steinberg, 1993). Whole cell Ca^{2+} currents were inhibited with nifedipine or cobalt and displayed strong inward currents when the membrane was depolarised. Similar L-type Ca^{2+} currents were observed in freshly isolated human, monkey and cultured foetal but not subcultures of human RPE cells (Ueda and Steinberg, 1995). These channels belong to the neuroendocrine subtype (Strauss et al., 2000). Cultured cells from RCS rats displayed an increased Ca^{2+} conductance and a depolarised V_M compared to normal rat RPE cells (Strauss and Wienrich, 1993). The reduced V_M would affect the activation of apical voltage-dependent K^+ channels (Hughes and Takahira, 1996) that are essential for fluid regulation of the subretinal space. The increased Ca^{2+} conductance may interfere with phagocytosis (Hall

et al., 1991). The L-type Ca^{2+} channels in RCS rats show altered regulation by PTKs and PKC (Mergler et al., 1998). L-type Ca^{2+} currents in cultured human and rat RPE cells show dual regulation by PTK and PKC, with the level of PKC activation determining whether PTK increases or decreases the L-type Ca^{2+} current (Strauss et al., 1997, 1999, 2000). This dual regulation was proposed as a potential role for L-type Ca^{2+} channel autoregulation of RPE by growth factors (Strauss et al., 1997). The altered integration of PTK and PKC in RCS rat RPE cells may underlie the retinal degeneration in this strain (Mergler et al., 1998). Strauss's group have identified that it is the membrane bound protein tyrosine kinase (pp60c-src) expressed in cultured RPE cells (Koh, 1992) that opens L-type Ca^{2+}-channels in cultured rat RPE cells by phosphorylation with the pore forming α_{1D} subunit (Strauss et al., 2000).

10.3. Internal Ca^{2+} stores

Owing to the multiple functions of free Ca^{2+} within the cell the $[Ca^{2+}]_{in}$ must be finely controlled, and the sequestration of calcium or the mobilisation of calcium stores is one mechanism by which this may be achieved. Ca^{2+} is stored within the ER and its regulation is important in considering the generation of the light rise. The conductance of ionic channels changes in less than a millisecond, and pumps and transporters respond in the time scale of seconds. In considering events with the duration of the EOG internal calcium changes are very likely to be important because they are related to the slow activation of second messenger signalling pathways at the inner surface membrane. Calcium is sequestrated in the ER, and its slow release could account for more of the delay in initiating the EOG light rise. Once released Ca^{2+} could gate open the basolateral Cl^- channel responsible for the light rise. Whilst there is no direct evidence that the ER is the source of Ca^{2+} the clinical evidence on the nature of the EOG demands that a totally intracellular mechanism determines the current and voltage changes (Arden and Wolf, 2000a).

Uptake of calcium into the ER is regulated by a calcium–ATPase pump (ERCA).[6] This is inactivated by the binding of a small unphosphorylated protein, phospholamban (Kirchberger et al., 1975). This is an extremely important point in the regulatory control of Ca^{2+}. The phosphorylation of phospholamban by various kinases operated on by second messengers exerts fine control over cytosolic free calcium.

Ca^{2+} release from the ER is dependent upon IP_3-R that forms an ionic Ca^{2+} channel. This is another example of the way the RPE is influenced by the extracellular fluid. The sequence is that when receptors on the RPE membrane combine with their specific targets, either a *G*-protein or tyrosine kinase is activated, and this in turn changes a pro-enzyme to a fully active isoform of phospholipase C (Nash and Osborne, 1996). This lipase hydrolyses the membrane-bound phospholipid (PIP_2) and IP_3 and DAG are formed. IP_3 can move in the cytosol to the ER where it binds to a receptor that opens a Ca^{2+} channel and Ca^{2+} is released into the cytosol. This release cuts short the activity of IP_3-R by negative feedback. For further reviews, see Bosanac et al. (2004), Berridge (1993), Ferris and Snyder. (1992).

11. Cystic fibrosis transmembrane conductance regulator

CF is a genetic disorder caused by several of the over 1000 mutations that have been identified in the gene encoding for CFTR (Rich et al., 1990). The gene responsible for CFTR has been localised to q31–q32 on the 7th chromosome (Riordan et al., 1989; Rommens et al., 1989). The intracellular portion of CFTR consists of two nucleotide binding domains (NBD1 and NBD2) separated by a large polar regulatory domain with nine sites for phosphorylation by PKA and seven sites for phosphorylation by PKC (Riordan et al., 1989). CFTR could be considered to be an ATP-gated Cl^- channel. CFTR is found in foetal RPE (Blaug et al., 2003), its' mRNA in adult RPE (Miller et al., 1992) where it transports Cl^- (Knowles et al., 1983). Only a fraction of the mutations cause known changes in function.[7] The predominant

[6]The calcium pump in the ER is inhibited by thapsigargin (Lytton et al., 1991) and/or cyclopiazonic acid (Kennedy and Mangini, 1996; Rymer et al., 2001; Seidler et al., 1989). These drugs deplete Ca^{2+} stores by inhibiting reuptake and are widely used to study Ca^{2+} regulation in the RPE (Kennedy and Mangini, 1996; Rymer et al., 2001). Physiologically, ERCA is inhibited by phospholamban in its unphosphorylated state (Tada and Kadoma, 1989). Phosphorylation of phospholamban by PKA-dependent cAMP thus provides a means of control of $[Ca^{2+}]$. Thus, the control of free calcium is regulated by cAMP and by $[Ca^{2+}]_{in}$ itself. Much of this work has been carried out in heart and skeletal muscle (Simmerman and Jones, 1998) as well as the RPE (Peterson et al., 1997; Rymer et al., 2001). Melanosomes also store calcium (Salceda and Riesgo-Escovar, 1990; Salceda and Sànchez-Chàvez, 2000) of the RPE. Melanin granules in the RPE have many important functions which are still under investigation although melanin does help to protect the RPE from oxidative stress,

(*footnote continued*)
lipofuscin accumulation and the degradation of rod outer segments. Melanosomes act as a further reservoir for intracellular Ca^{2+} (Drager, 1985; Panessa and Zadunaisky, 1981). Uptake of Ca^{2+} by melanosomes is independent of ATP and probably relies upon a Ca^{2+}–H^+ exchanger. See reviews by Peters and Schraermeyer (2001) and Schraermeyer and Heimann (1999) for further details.

[7]The gene for CFTR is found on chromosome 7 that encodes for a protein, CFTR, consisting of 1480 amino acids forming two transmembrane spanning regions of six α-helixes, with minimal exposure to the extracellular space. Various classes of malformation occur. Class 1 results in the malformation of CFTR so that it does not reach the Golgi apparatus. In class II, the protein cannot leave the ER and consequently there is an absence or severe depletion of CFTR at the plasma membrane. In class III, the gating of the channel is affected with the channel failing to open following cAMP stimulation (Logan et al., 1994). In class IV the conductance of the pore formed by the protein is reduced (Ashcroft, 2000; Sheppard et al., 1993; Welsh and Smith, 1993). CFTR belongs to the large ATP-binding cassette family of transporters that actively transport a variety of substrates and ions (Higgins, 1992; Stefkova et al., 2004). The frequency of CFTR mutations varies between ethnic groups and countries

mutation (Δ508) in Caucasian CF is a deletion of phenylalanine in NBD1 (Cutting et al., 1990; Lemna et al., 1990) that results in a CFTR unable to reach the plasma membrane (Puchelle et al., 1992). Therefore it is degraded before reaching the Golgi (Yang et al., 1993). The sequence of ATP-binding, hydrolysis and the interaction of the R-domain and how these components interact to regulate gating of CFTR have been debated by various authors (Aleksandrov et al., 2002; Anderson et al., 1991a; Baukrowitz et al., 1994; Gadsby and Nairn, 1999; Gunderson and Kopito, 1995; Winter and Welsh, 1997). Evidence now suggests that when ATP binds to NBD2 the two NBDs become protonated and form a dimer and this opens the pore. Hydrolysis of ATP at NBD2 closes the pore and disrupts the dimer (Vergani et al., 2005). Gating of CFTR has recently been reviewed by Riordan (2005) and Linsdell (2006). Phosphorylation of the regulatory domain by PKA is also required to prime the channel for opening (Li et al., 1988). CFTR currents are non-rectifying when Cl^- concentrations are symmetrical across the membrane (Anderson et al., 1991b, c; Kartner et al., 1991). The halide permeability sequence is now thought to be $I^- > Br^- > Cl^- > F^-$ (Tabcharani et al., 1997).

11.1. Role of CFTR in the RPE

Functional CFTR has not been demonstrated directly in adult human RPE. However, CFTR protein is expressed in cultured adult human RPE cells (Weng et al., 2002) as well as in the transfected human RPE foetal cell line (Wills et al., 2000). It has been localised to the basal and apical membranes in primary human foetal RPE explants (Blaug et al., 2003) and its mRNA identified in both retina and RPE from donor human and bovine eyes (Miller et al., 1992). It is also functionally expressed in the human RPE cell line, ARPE-19 (Reigada and Mitchell, 2005). CFTR is thought to play a role in fluid regulation by the RPE by because it can transport Cl^- (Blaug et al., 2003). However, the bulk of fluid regulation in the RPE is performed by calcium-gated Cl^- channels and the aquaporins, and if CFTR was significantly involved in fluid regulation then it would be expected that CF individuals would be prone to macula oedema (Loewen et al., 2003) which is not observed. However, CFTR could produce current and voltage changes across the RPE and transport molecules through the membrane which could affect other conductances as well as potentially playing a role in the regulation of pH.[8]

11.1.1. Ionic channel interactions

CFTR has numerous interactions with other ionic channels in epithelia and therefore a similar function may exist in the RPE. Control epithelia (from the nose, lung and cell line models) "absorb" less sodium than in affected epithelia lacking CFTR. These effects have been demonstrated to be due to a direct inhibitory action of CFTR on an amiloride sensitive epithelial Na^+ channels (Boucher et al., 1986; Ismailov et al., 1996; Stutts et al., 1995). Activation of inward rectifying K^+ channels has also been demonstrated by cAMP in the presence of functional CFTR (Loussouarn et al., 1996). Furthermore, regulation of an outward rectifying anion channel by CFTR has been demonstrated in planar lipid bilayers (Jovov et al., 1995). In hypotonic solutions, CFTR releases ATP into the extracellular compartment. ATP can then bind to the purinergic receptor P_2Y, which would then cause a rise in $[Ca^{2+}]_{in}$ (Braunstein et al., 2004; Prat et al., 1996; Reisin

(*footnote continued*)
and individuals with identical genotypes may not have similar phenotypes, indicating a role for environmental factors in determining the final severity of CF (Duguépéroux and De Braekeleer, 2004; Estivill et al., 1997).

Blocking of CFTR currents has proven difficult with organic compounds. Whilst DIDS inhibit CFTR currents in a voltage-dependent manner when applied to the cytoplasmic face, they are ineffective when applied to the extracellular face (Anderson et al., 1991b; Cliff and Frizzell, 1990; Linsdell and Hanrahan, 1996). Partial inhibition is possible with millimolar concentrations of diphenylamine-2-carboxylic acid (DPC) and flufenamic acid from the extracellular surface (McCarty et al., 1993). However, the blockage is voltage dependent and not complete. Native but not synthetic scorpion toxin when applied to the cytoplasmic face is an effective blocker, independent of voltage (Fuller et al., 2004). Substrates of the closely related 'multidrug resistance protein' such as taurolithocholate-3-sulphate and β-estradiol also block CFTR currents in a voltage-dependent manner when applied to the cytoplasmic surface (Linsdell and Hanrahan, 1999) and glibenclamide has similar effects (Sheppard and Robinson, 1997) apparently obstructing the open pore (Zhang et al., 2004). However, glibenclamide also inhibits K^+ channels (Yamazaki and Hume, 1997). In contrast, $CFTR_{inh}$-172 is a small thiazolidinone molecule that blocks CFTR by prolonging the mean channel closed time independently of membrane voltage. It is suspected to exert its action by binding to NBD1 (Ma et al., 2002; Muanprasat et al., 2004; Taddei et al., 2004) and has been used to study CFTR in an RPE cell line (Reigada and Mitchell, 2005).

[8]CFTR has a major role in regulating the intracellular pH as well as the pH of the mucosal membranes of the gut (Kopelman et al., 1988). In the gut the mechanism is accomplished by CFTR maintaining a favourable $[Cl^-]_{out}$ which maintains the $Cl^-_{in}/HCO^-_{3\,out}$ exchanger to regulate pH_{in} (Jetten et al., 1989; Paradiso et al., 2003; Simpson et al., 2005) whilst in the lungs CFTR apparently controls a direct HCO_3^- conductance (Coakley et al., 2003), which accounts for the acidic nature of the airway mucosa in CF sufferers. CFTR demonstrates mild permeability to HCO_3^- under non-physiological conditions (Gray et al., 1990; Linsdell et al., 1997b). Whole cell recordings of voltage, current and intracellular pH_{in} from CFTR protein expressed in *Xenopus* oocytes show a switch of conductance from Cl^- only to HCO_3^- and Cl^- when $[Cl^-]_{out}$ is reduced to 0–10 mM (Shcheynikov et al., 2004). This switch may help to control pH_{out} when there are rapid changes in $[Cl^-]_{out}$. In the pancreas, CFTR is inhibited by reduction in $[HCO_3^-]_{out}$ (O'Reilly et al., 2000). In amphibian RPE pH_{in} is the main contributor to fluid transport that stimulates a basolateral HCO_3^-/Cl^- exchanger (Lin and Miller, 1994). Whether CFTR plays a role in regulating pH_{in} in mammalian RPE is unknown. But it has properties that would enable it to regulate intracellular pH: it transports carbonate and chloride ions, to regulate extracellular pH in a variety of epithelia (Akiba et al., 2005; Anderson et al., 1991c; Hug et al., 2003; Paradiso et al., 2003; Poulsen et al., 1994; Ulrich, 2000, Wang et al., 2005). Although in amphibians the RPE chloride conductance is DIDS-sensitive, there may be a species difference that renders mammalian chloride conductance insensitive to DIDS applied from the extracellular space (Hughes and Segawa, 1993; Linsdell and Hanrahan, 1996). In this case CFTR could account for (some) portion of the EOG.

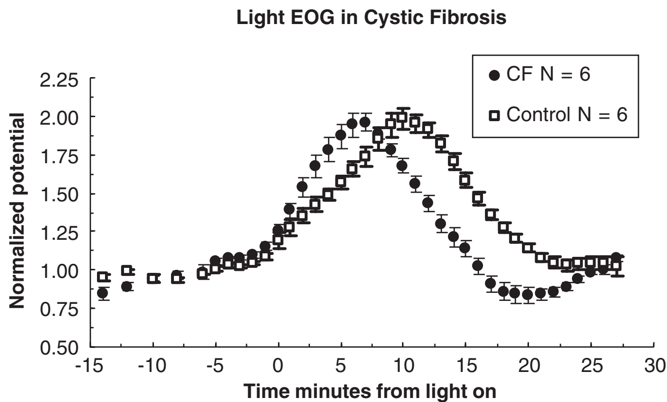

Fig. 7. Preliminary findings of the light-EOG in a group of individuals with CF ($N = 6$) and controls ($N = 6$). Light was 100 cd/m^2 with dilated pupils at $t = 0$. Graphs are mean±SEM. Three were homozygous and three were heterozygous for Δ508. The amplitude of the light rise is normal, however the time to peak is significantly faster ($p<0.001$) than normal. Data normalised to the mean voltage of the 10 min preceding the stimulus. The absolute peak value = 1.98±0.07 for controls and 1.96±0.06 for CF patients.

et al., 1994; Taylor et al., 1998). In turn this change of calcium would increase Cl⁻ fluxes, (Schwiebert et al., 1995). This may be a general means of regulating cell volume. Findings on work using bovine RPE and a human RPE cell line also suggest that CFTR increases ATP in the subretinal space following activation (Mitchell, 2001; Reigada and Mitchell, 2005).

The mechanism by which CFTR facilitates release or transports ATP across the membrane has not been fully resolved. ATP release from cells has not been universally supported by all investigators (Abraham et al., 1997; Grygorczyk and Hanrahan, 1997b; Li et al., 1996; Reddy et al., 1996) with the possibility that the ATP released from the cell is a result of mechanical disruption of the plasma membrane during patch formation (Grygorczyk and Hanrahan, 1997a).

If CFTR played an important role in generating the light rise, then individuals with CF should have a reduced response which is not the case (Lara et al., 2003; Miller et al., 1992) (see also Fig. 7 showing the normal light rise amplitude in a group of individuals with CF). However, we observed an altered time course of the light rise in this group of CF volunteers comprising three heterozygous and three homozygotes for the Δ508 mutation. This may implicate CFTR in determining the time course of the light-EOG and therefore a role for CFTR in the light rise cannot be excluded. One alternative explanation could be that another Cl⁻ transporter is up-regulated when CFTR is defective and therefore takes over its function. For further reviews in CFTR's interactions, see Kunzelmann (2001) and Schwiebert et al. (1999).

11.2. ClC chloride channels

These channels are very common and various sub-groupings have been described. ClC-2 is found in the RPE, and in a ClC-2 knockout mouse, retinal degeneration and testicular atrophy were the only defects (Bösl et al., 2001; Nehrke et al., 2002). It is highly likely that ClC-2 in the RPE plays a vital role in either the regulation of cell volume, pH_{out} and fluid secretion as it does in other epithelia (Cuppoletti et al., 1993; Furukawa et al., 1998; Malinowska et al., 1995). ClC-2, ClC-3, ClC-5 and CFTR are co-expressed in human foetal RPE cell line (Wills et al., 2000) and as CFTR and ClC-2 are activated by PKA (Anderson et al., 1991a; Cid et al., 1995; Tewari et al., 2000) ClC-2 may provide an alternative Cl⁻ conductance when CFTR is defective (Blaisdell et al., 2000; Cid et al., 1995; Jordt and Jentsch, 1997; Schwiebert et al., 1998; Thiemann et al., 1992). However, this proposal is not supported by recent evidence. In a doubly deficient mouse, with both CFTR and ClC-2 disrupted, the colonic epithelial Cl⁻ currents were not reduced (Zdebik et al., 2004) casting doubt on the role of ClC-2 as a rescue channel for CFTR.[9]

12. Channel changes associated with the clinical EOG

12.1. The fast oscillation

The FO is normal in Best's whilst it is abnormal in retinitis pigmentosa (RP) and therefore the generation of the light rise and the FOs are different (Sandbach and Vaegan, 2003; Vaegan, 1993; Vaegan and Beaumont, 1996, 2005; Weleber, 1989) (see Fig. 8). One model attributes the difference as indicating that the light rise is dependent upon the bestrophin Cl⁻ channel and the FOs relying on CFTR (Blaug et al., 2003; Miller et al., 1992).

[9] ClC Cl⁻ channels were first identified in the electric organ of the *Torpedo* ray (White and Miller, 1979) since named (ClC-0) (Jentsch et al., 1990). A further nine ClC channels have been identified in mammals. They activate slowly at hyperpolarising voltage and conduct anions preferentially in the sequence, $Cl^- \geqslant Br^- > I^-$ (Thiemann et al., 1992). The diversity of ClC-2 distribution implies that it has a key role to play in epithelial and non-epithelial cells. ClC-2 is activated by acidification of the extracellular space (Jordt and Jentsch, 1997) and is typically closed at resting membrane potential. Its main role seems to be in regulating cell volume (Furukawa et al., 1998; Gründer et al., 1992; Strange et al., 1996; Xiong et al., 1999).The X-ray structure of ClC channels has been described in prokaryotes (Dutzler et al., 2002) with the channel comprising 18 α-helixes running anti-parallel with the positive N terminus orientated towards the centre creating a domain to increase anion binding and gated closed by glutamate residues that mimic Cl⁻ (Dutzler, 2004). ClC Cl⁻ channels have two pores that are identical in structure that open in bursts with each pore fluctuating between open and closed (Hanke and Miller, 1983; Miller, 1982). The gating and selectivity of ClC Cl⁻ channels is still unresolved, although structural models have been ascertained for bacterial ClC channels (Pirruccello et al., 2002).

ClC-2, ClC-5 and ClC-3 mRNA and immunocytochemical techniques have identified these channels in a transfected human foetal RPE cell line (Wills et al., 2000). The electrophysiology of the different types of ClC channel is not identical. ClC-2 has slight inward rectification in the range from −180 to 50 mV that is slowly activated both by hyperpolarisation, extracellular acidification, PKA and arachidonic acid (Cuppoletti et al., 1993; Tewari et al., 2000). ClC-2 is blocked by DPC and 9-anthracene-carboxlic acid (Gründer et al., 1992; Thiemann et al., 1992).

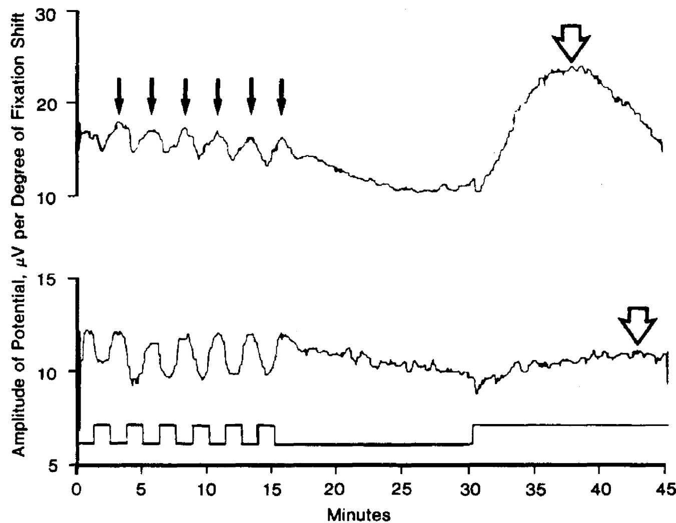

Fig. 8. Upper trace showing normal fast oscillations (arrows) and light rise (broad arrow). Lower trace shows the absence of the light rise in Best's macular dystrophy. From Weleber (1989). Reprinted with permission of Archives of Ophthalmology.

Figs. 9A and B show recordings of the FOs from a normal and Δ508 homozygous volunteers. It can be seen that the standing potential rises in the dark and falls in the light. The minimum occurs 30–45 s after light onset and then the standing potential begins to rise once more and with repeated dark–light periods a series of oscillations can be established.

The model which is generally accepted at the time of writing may not be complete but the first step is the photoreceptor-induced decrease in $[K^+]_{out}$ in the subretinal space. This opens voltage-gated potassium channels in the apical membrane of the RPE (and also in the Müller cells) that results in the c-wave of the ERG (Linsenmeier and Steinberg, 1983). The activity of the Na–K–2Cl exchanger decreases as $[K^+]_{out}$ is reduced (Joseph and Miller, 1991). Because now the inward flux caused by the Na–K–2Cl transporter is reduced, the intracellular Cl^- activity decreases (Gallemore and Steinberg, 1993). Consequently, the CFTR Cl^- current also decreases and therefore the basal membrane of the RPE hyperpolarises, leading to a

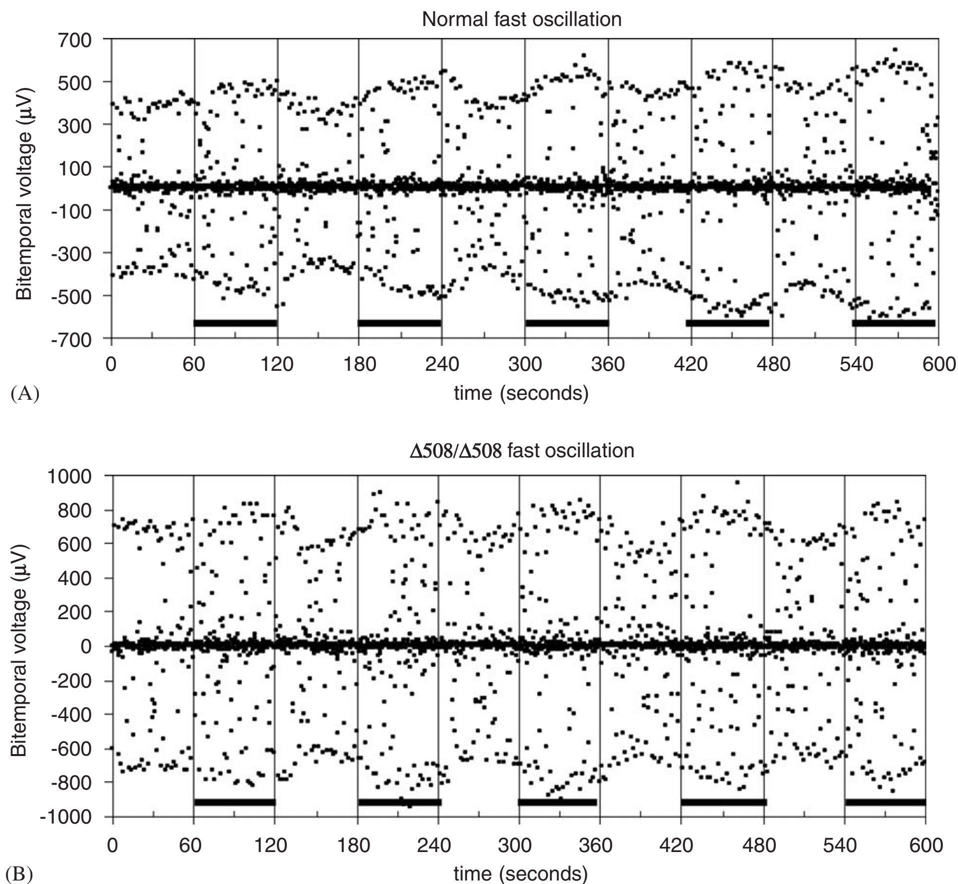

Fig. 9. (A) The fast oscillation (FO) recorded in a 20-year old female over 10 min with alternating periods of light and dark at 1 min intervals. We used bi-temporal skin electrodes with alternating periods of light (100 cd/m^2) and dark (0.01 cd/m^2) at 1 min intervals. Saccades were performed at 1 Hz and the unfiltered raw bi-temporal voltages were sampled continuously at 4 Hz using a simple digital voltmeter. The first 60 s was in light. When the illumination is reduced (dark bar) there is a rise in the standing potential (dark rise) that peaks at ~30 s. When illumination is restored there is a decrease in the standing potential leading to the "light trough" that reaches a minimum after ~30 s and then begins to rise. In this study, we took the mean of four dark–light cycles of the peak-trough voltages giving the dark rise : light trough ratio (DR:LT). In this individual the DR:LT ratio was 1.29 ± 0.06 (mean ± SD). (B) The FO recorded in an 18-year old female with the Δ508/Δ508 genotype for CF. Mean ratio of the DR:LT was 1.35 ± 0.05 (mean ± SD). Note the similarity between A and B. although the Δ508 mutation has been associated with a reduced FO (see text).

fall in the TEP following light onset and this generates the light trough of the FO of the EOG. This model is based upon the work of Blaug et al. (2003) who used human foetal RPE sheets mounted in an Ussing chamber where CFTR expression was demonstrated to support the role of CFTR in the FO light trough. When the effect of light was simulated by reducing the apical bath K^+ level from 5 to 2 mM, in the absence of cAMP, V_{Apical} and V_{Basal} change together with V_{Apical} hyperpolarising at a greater rate than V_{Basal} so the TEP transiently increases, as a "mock c-wave" develops. In the next phase, V_{Basal} hyperpolarises at a greater rate than V_{Apical} and so the TEP falls which is a result of a reduction in $[Cl^-]_{in}$ due to a reduced flux through the Na–K–2Cl cotransporter (Joseph and Miller, 1991). In a tissue treated to increase cAMP levels, the same procedure causes the same sequence but there is a dramatic increase in the magnitude and time course of the FO trough which suggests that CFTR is involved in generating the FO. This model is supported by the reports that in CF the amplitudes of the FO are reduced and delayed whilst the light-EOG remains normal (Lara et al., 2003; Miller et al., 1992). Whilst Blaug could show that elevating intracellular cAMP increased the amplitude of the FOs in foetal RPE sheets where the mRNA and immunocytochemical evidence supported the presence of CFTR he was at that time unable to block these changes using a specific CFTR inhibitor. Therefore, these changes may have been due to another Cl^- channel that is also gated by elevations in cAMP such as ClC-2 (Bösl et al., 2001; Cuppoletti et al., 2000).

However, there is also evidence against this model. Reduction of intracellular Cl^- $[Cl^-]_{in}$ does *not* affect wild type-CFTR or Δ508CFTR Cl^- currents whilst in transfected cell lines the reduction of $[Cl^-]_{out}$ reduces chloride current (Wright et al., 2004). In addition, the reduction of the FO reported in CFTR may not be universal. None of a small group ($N = 3$) of individuals homozygous for Δ508 mutations showed reduced FOs whilst a slight delay was observed (Constable et al., 2005)—see Fig. 9. There are clearly some contradictions regarding CFTR, ATP and the light rise. Certainly CFTR mediates release of ATP at the apical membrane from bovine and human RPE cell lines (Reigada and Mitchell, 2005) and that stimulation of P_2Y_2 receptors by ATP elevates $[Ca^{2+}]_{in}$ (Collison et al., 2005; Peterson et al., 1997; Sullivan et al., 1997). It is also the case that the rise in $[Ca^{2+}]_{in}$ opens the basolateral Cl^- channel involved in the slower light rise of the EOG. Therefore, this model predicts that the light rise will be affected when CFTR is defective or absent which is not the case (Lara et al., 2003; Miller et al., 1992) and Fig. 7 above. Therefore, we must conclude that this simple model is incomplete. However, all three assumptions are well supported by data but further work will be required to determine precisely the role that CFTR plays in the light rise and FOs. Fig. 10 illustrates the current model of the FO generation and the light rise.

13. The elusive light rise substance

Our current understanding of the light rise is basically the model produced by Steinberg: light liberates a substance from the rods that binds to a receptor in the apical surface of the RPE. More recent work has added that second signalling systems inside the cytosol produce a rise in intracellular $[Ca^{2+}]$ which in turn increases the basolateral Cl^- conductance demonstrated by Steinberg. The identity of the substance, the means by which it increases $[Ca^{2+}]_{in}$ and whether the ultimate basolateral Cl^- channel is bestrophin or CaCC is unknown. Ethanol can generate a sequence of slow changes to the human EOG that are identical to that produced by light, implying that the time course is generated internally within the RPE. Light and alcohol produced current changes that share a final common pathway, again supporting the idea that the light rise is mediated via a Ca^{2+} pathway (and see below).

Work on the membrane and cytosol characteristics of RPE cells has mostly been carried out in tissue culture and in various animal models that show some species differences. Therefore, any link between changes in the membrane properties associated with a particular agent and the light rise is speculative, unless a selective blocking agent can be found, that, used in vivo, affects the EOG in predictable ways (Eggermont, 2004; Nilius and Droogmans, 2003). Another difficulty encountered is the difference between RPE cells of different species. For example, with elevation of intracellular cAMP in bovine preparations (Rymer et al., 2001) and chick (Kuntz et al., 1994) there is a decrease in basolateral Cl^- conductance whilst in human foetal RPE (Quinn et al., 2001) as well as whole cell recordings from cultured human RPE (Weng et al., 2002) basolateral Cl^- conductance increases. In frog RPE, cAMP-activated Cl^- currents are inhibited by extracellular DIDS (Hughes and Segawa, 1993) unlike the comparable mammalian cAMP activated Cl^- current generated by CFTR (Linsdell and Hanrahan, 1996; Schultz et al., 1999).

Whilst the RCS rat model of retinal degeneration have provided valuable insights into the regulation of Ca^{2+} currents that closely resemble human (Mergler et al., 1998; Ueda and Steinberg, 1994, 1995) there is some dispute about the identity of bestrophin as the Ca^{2+} activated Cl^- channel involved in the generation of the light rise (Marmorstein et al., 2004; Pollack et al., 2005; Strauss and Rosenthal, 2005). Until a demonstrable knock-out animal model of the light rise can be associated with bestrophin and then restored, the question as to whether bestrophin is the CaCC responsible for the light rise will remain in doubt (Qu et al., 2004; Qu and Hartzell, 2004; Rosenthal et al., 2005; Strauss and Rosenthal, 2005). Although evidence is now stronger that bestrophin is a CaCC and not a modulator of a separate Cl^- channel (Fischmeister and Hartzell, 2005; Hartzell et al., 2005b).

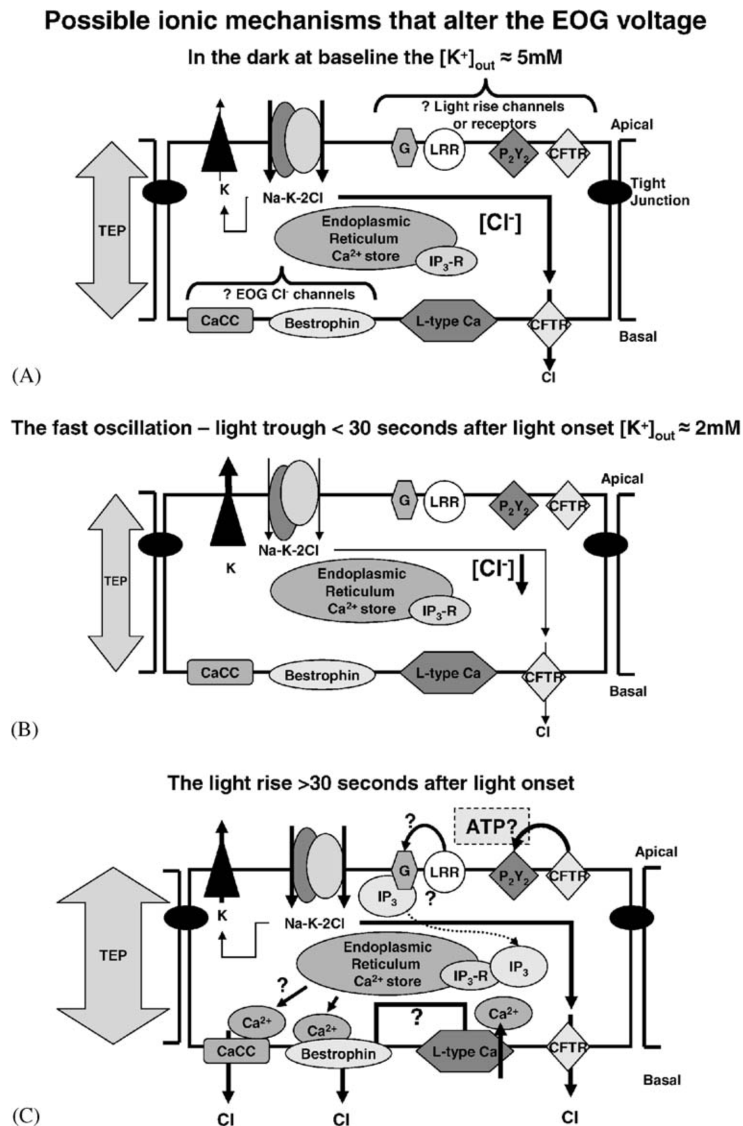

Fig. 10. Schematic representations of the ionic channels and signalling molecules involved in the generation of the fast oscillation (FO) and the light rise in man. At the apical membrane, the possible ionic channels and receptors involved in initiating or generating the light evoked responses are grouped together. These are: a proposed light rise receptor (LRR) that may be coupled to a *G*-protein or the purinergic receptor (P_2Y_2). At the basal membrane the chloride channels that have been proposed for altering V_{Basal} are grouped together. The light rise Cl^- channel may be either a CaCC or bestrophin. However, the mechanism may be more complex, and the channel may interact with a basolateral Ca^{2+} channel (possibly an L-type) to elevate $[Ca^{2+}]_{in}$. There are several possible mechanisms that have been proposed and we are still unsure as to the exact nature of the intermediary steps between light onset and the final depolarisation of V_{Basal} that generates the light rise. (A) The upper figure shows the RPE cell in the dark where subretinal $[K^+]$ is maintained by the photoreceptor dark current. This high $[K^+]$ provides a favourable concentration gradient for the entry of Na^+, K^+ and Cl^- ions via the apical cotransporter. (B) In light, the dark current is not present and the subretinal $[K^+]$ falls. The RPE K^+ now moves down its electrochemical gradient through apical voltage-gated K^+ channels into the subretinal space. The Na–K–2Cl cotransporter has a reduced transport and so intracellular $[Cl^-]$ falls owing to the transiently decreased subretinal $[K^+]$. This then reduces the active transport of Cl^- ions by the cystic fibrosis transmembrane conductance regulator (CFTR) so the basal membrane hyperpolarises and causes a fall in the TEP. This is seen as the light trough of the FO. (C) Some possible pathways for the generation of the light rise. The putative light rise receptor LRR is situated on the apical membrane. The diagram suggests that LRR is satisfied by ATP. This may be provided by CFTR releasing ATP. Alternatively, the source of ATP may originate from the rods. Activation of either LRR or the P_2Y_2 receptor initiates the rise in $[Ca^{2+}]_{in}$ and this is presumed to be the second messenger involved in the light rise (see text). The source of Ca^{2+} may derive from the endoplasmic reticulum via the generation of inositol triphosphate (IP_3) that binds to the IP_3-R receptor. An alternative hypothesis is that the source of Ca^{2+} derives from an interaction of bestrophin with an L-type Ca^{2+} channel that facilitates the entry of Ca^{2+} across the basolateral membrane. In this model bestrophin is not the basolateral Cl^- channel responsible but a distinct CaCC is finally responsible for the light rise. The multiple steps in the generation of the light rise accounts for its slow onset of ~60 s with a peak at ~8 min.

13.1. Some possible candidates

At the apical membrane of the RPE there are various receptors that could conceivably mediate the light rise. Dopamine was considered a possible candidate for the light rise substance because the light rise was reduced in depressives but elevated in manic depressives (Economou and Stefanis, 1979). However, in vitro

studies have been unable to conclusively demonstrate that dopamine is the light rise substance (Dawis and Niemeyer, 1986; Gallemore and Steinberg, 1990; Rudolf and Wioland, 1990; Textorius et al., 1989) despite dopamine increasing in the subretinal space following light onset (Kramer, 1971).

Adrenergic, muscarinic, neuropeptidic and purinergic receptors have been identified at the apical membrane of the RPE in a variety of species and in cell culture (Ammar et al., 1998; Collison et al., 2005; Crook et al., 1992; Frambach et al., 1990; Mitchell, 2001; Peterson et al., 1997; Quinn et al., 2001; Rymer et al., 2001) and despite their potential to elevate $[Ca^{2+}]_{in}$ no definitive light rise receptor or substance has been found. However, the purinergic receptor (P_2Y_2) (Sullivan et al., 1997) with ATP as the agonist has recently been postulated to be involved (Reigada and Mitchell, 2005).

We speculate that ATP may be the light rise substance although the source may not be from the RPE as originally modelled (Reigada and Mitchell, 2005). In light, ATP is released by glial cells (Newman, 2003) that could diffuse towards the RPE. Alternatively, when ATP is degraded to adenosine, in the presence of ATP adenosine receptors on the RPE might evoke a rise in $[Ca^{2+}]_{in}$ (Collison et al., 2005). Another possibility is that ATP released from photoreceptors could be the light rise substance (Uehara et al., 1990). Because the rods consume very large amounts of ATP (Hagins, 1972) to develop the dark current the oxygen tension in the inner limbs falls to zero in darkness. The lactate profile across rat retina does not alter significantly and the rate of production is virtually unaltered when going from light to darkness (Winkler et al., 2003). Consequently, any store of ATP in the rod must be very small. However, when illuminated, the non-selective channels in the outer rod limb close very rapidly, while the Na–K–ATPase exchange pump continues to operate at high speed for some seconds, causing potassium depletion in the subretinal space. It soon stops, but it is plausible that the production of ATP continues for an additional further period so the intracellular ATP concentration of the rod rises: ATP then escaping into the subretinal space could provide the trigger for the light rise. This idea is attractive because it explains certain peculiarities of the EOG which have been known for many years (Arden, 1962; Arden and Kelsey, 1962b). For example, dark adaptation must precede the light rise, but the relation between the duration of preceding darkness and the size of the light rise does not conform to either psychophysical threshold changes or densitometric measures of rhodopsin regeneration. Again, although the rods produce the signal that influences the RPE, rod function can be normal when the EOG is reduced. With the multitude of integration sites for cross-talk between signalling molecules and receptors (Nash and Osborne, 1996) a more complex picture of the light rise will undoubtedly evolve as our understanding of the RPE–retina complex increases.

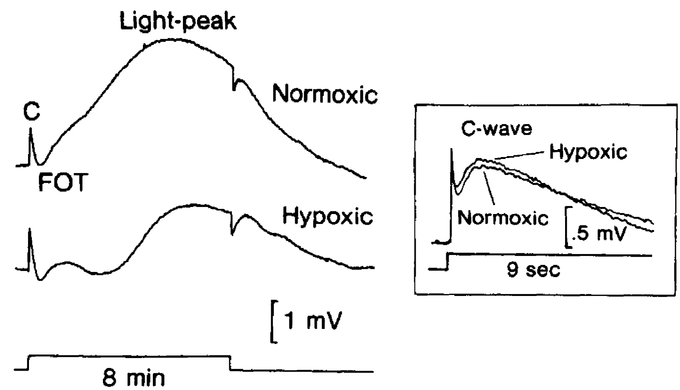

Fig. 11. Hypoxia decreases the light rise in cat (Linsenmeier and Steinberg, 1986). Reprinted with the permission of Investigative Ophthalmology and Visual Science.

14. Physiological characteristics and pharmacology of the light-EOG

The pharmacology of the DC potential, its relation to neurotransmitters and its reliance on changes in metabolism have been investigated by several authors. The slow light peak is extremely sensitive to anoxia (Kreienbuhl and Niemeyer, 1985; Linsenmeier et al., 1983, 1987; Linsenmeier and Steinberg, 1986; Steinberg, 1987). Fig. 11 shows that in mild experimental hypoxia, which does not affect the ERG, the light rise is markedly reduced and similar clinical findings have been reported. (Arden, 1962; Arden et al., 1962; Arden and Kelsey, 1962a; Niemeyer and Steinberg, 1984). Small changes in pH and pCO_2 also dramatically reduce the amplitude of the slow changes in potential caused by light. In view of the complex acid–base regulatory systems described above, it is not surprising that CO_2 and acidification exert differential effects on the TEP and consequently the corneo-fundal standing potential (Dawis et al., 1985; Niemeyer and Steinberg, 1984).

Acetazolamide (Kawasaki et al., 1986; Madachi-Yamamoto et al., 1984a) and changes in osmolarity (Kawasaki et al., 1984; Madachi-Yamamoto et al., 1984b) cause a slow reduction in the EOG voltages, and these findings are the basis on which clinical tests have been developed (see below). In addition, the potentials are influenced by biogenic amines and other substances (Bialek et al., 1996; Dawis and Niemeyer, 1986; Edelman and Miller, 1991; Joseph and Miller, 1992; Rymer et al., 2001; Textorius et al., 1989). Recently, it has been shown that ethyl alcohol affects the human corneo-fundal potential (Arden and Wolf, 2000a, b; Arden et al., 2000; Skoog et al., 1975; Wolf and Arden, 2004). Also at very low dosage non-steroidal anti-inflammatory drugs alter the TEP in isolated porcine and bovine preparations (Arndt et al., 2001; Bialek et al., 1996), and, in doses higher than clinically advisable, they affect the human EOG (Arden, unpublished).

Table 1
Some values reported for the FO ratio of the dark: light amplitudes with luminance

Author	Dark rise : light trough ratio	N	Luminance (cd/m^2)	Stimulus duration (s)
Vaegan (personal communication)	1.16 ± 0.08	58	200	60
Mergaerts et al. (2001)	1.13 ± 0.01	51	250	60
Miller et al. (1992)	1.26 ± 0.09	15	—	—
Weleber (1989)	1.22 ± 0.15	8	70	75
Schneck et al. (2000)	1.20 ± 0.03	9	30	60
Constable et al. (2005)	1.28 ± 0.05	8	100	60

Original units have been converted to cd/m^2 and rounded off. There is some variation between authors that may be a result of differing analysis of the FOs (see text).

14.1. Practical considerations in recording the fast oscillation

The FO is an important test that is rapid to perform and should be conducted in conjunction with the light-EOG. The reason is that the FOs and light rise are often selectively affected as in RP and Best's (Sandbach and Vaegan, 2003; Vaegan, 1993; Vaegan and Beaumont, 2005; Weleber, 1989) (see Fig. 8). Furthermore, a normal FO in the presence of an abnormal light rise may give the clinician greater confidence in the results obtained. In small studies, the FO is reported to be reduced in retinal vein occlusion (Rohde et al., 1981), rod monochromatism (Thaler et al., 1986), gyrate atrophy, pathologic myopia and Goldmann Favre syndrome (Vaegan and Beaumont, 2005). There is some variation between different authors' practices when measurements of the FO are reported. The International Society for Clinical Electrophysiology of Vision (ISCEV) standard for the FO recommendations are (1) six light–dark cycles lasting 120–160 s each should be employed (2) the average peak to trough ratio be used, and (3) the average latency or phase shift of the peaks should be recorded and (4) the absolute magnitude of the standing potential in the troughs (in microvolts per degree of visual angle) be recorded (Marmor and Zrenner, 1993). These recommendations are not always followed. Two large studies by Mergaerts et al. (2001) and Vaegan and Beaumont (2005) investigated the nature of the FO in man. Both found that the FO amplitudes were independent of age, sex and that four cycles of light and dark was sufficient to evaluate the FO parameters. The former investigators recorded the difference between maximum in dark and minimum in light as $69.6 \pm 5.3\,\mu V$, and the ratio dark/light 1.112 ± 0.013 (Mergaerts et al., 2001). Another method of analysis has been to fit a sine wave to the data and determine the period and amplitude of the variation (Weleber, 1989). In a small study, Thaler et al. (1982) found a linear relationship between FO amplitudes and luminance (8–955 cd/m^2). The duration of the preceding dark adaptation had no effect on the amplitude. The results of various investigations into the normal dark rise: light trough (DR:LT) ratios are shown below in Table 1. Vaegan and colleagues have investigated the FO amplitudes in a large series of normals ($N = 58$) and found the lower limit of normal (5% point) to be 1.05 (Sandbach and Vaegan, 2003). The lower mean values reported by Mergaerts et al. (2001), and Vaegan may be attributed to the data analysis where the authors averaged to total amplitudes in the light and dark intervals rather than measuring the peak to trough ratio which yields an approximately normal value between 1.2 and 1.3 (Constable et al., 2005; Miller et al., 1992; Schneck et al., 2000; Weleber, 1989). The brighter backgrounds used by some authors is also unlikely to be the cause of the lower ratios as the FO increases with light intensity and therefore the ratios should be constant (Thaler et al., 1982).

The FOs can be easily recorded as part of the clinical light-EOG procedure. The level of retinal illumination that precedes the standard test is usually uncontrolled. The FOs can be recorded confidently using a minimum of three cycles of light/dark, each lasting 2 min (Vaegan and Beaumont, 2005). If a determination of the FOs is carried out before the 12 min of dark adaptation that precedes the EOG, the FO measurement will ensure a constant level of light adaptation from which the EOG itself can be measured. This should reduce variability in the light rise and the time penalty incurred is small (Vaegan and Beaumont, 2005).

The amplitude of the FO can be increased by raised blood glucose levels; however, whether this change differs in diabetics and non-diabetics is still to be evaluated but may represent the sensitivity of the FO to RPE metabolism (Schneck et al., 2000). Given the relative ease of recording the FO and its specificity for RP this procedure would appear to be a useful adjunct to the light-EOG (Vaegan and Beaumont, 1996).

15. Recording the EOG

An ISCEV standard (currently under review) gives recommendations for standard clinical tests (Marmor and Zrenner, 1993). The eye movement potential is easily recorded with skin electrodes placed one on either side of the eye. The voltage varies considerably depending upon how close the electrodes are placed to the eye, but 12–30 μV per degree is usual. Rapid saccadic eye movements may be

made over 30°, so the voltage change recorded to such eye movements is ~1 mV. This relatively large signal makes the EOG test technically undemanding. Eye movements >30° are not desirable, because they tend to be carried out in two or more saccades, making measurement of the voltage difficult or impossible with AC recording techniques. Horizontal eye movements should be made, because otherwise, artefactual voltages associated with lid elevation may also be recorded. Direct recording of the DC voltage between the two electrodes is possible, but changes in polarisation of the electrodes, and slow changes in skin potentials occur, and interpretation of such slow voltage changes without eye movement is impossible in clinical conditions. With AC-coupled amplifiers, recording eye movement voltages, any type of surface electrode can be used, including disposable Ag/AgCl pads, or gold cup electrodes. It is desirable to lightly abrade the skin on which the electrodes are placed, to reduce contact resistance below 5 kΩ.

The eye movements can be made in any way convenient. Some workers have advocated that the subject is given two (red) fixation points and asked to look left and right between them, at any convenient rate. Other workers expose only one fixation point at a time, and alternate the fixation points at a suitable rate which should be constant throughout the test. Patients are most comfortable with between one and two eye movements per second. More elaborate schemes have been proposed. For example, a number of closely spaced fixation points have been used, lit sequentially, and the subject is asked to follow the moving spot. The fixation points are so spaced that the angle of gaze varies sinusoidally with time. The amplitude of the voltage change can be determined precisely by a suitable software package (Fourier analyser). In the authors' experience, the simplest method is preferable.

15.1. Technical difficulties

These are very rare with the EOG. In a very few cases, patients may find difficulty in making standard eye movements. These include cases of ophthalmoplegia, or nystagmus (muscle paralyses, Parkinsonism, myasthenia). In certain of these cases, the patient may attempt to compensate by moving the head, not the eye. Such problems can usually be overcome by providing a solid, comfortable head rest, and encouraging the patient to relax the neck muscles. When the patient is first instructed about how to do the test, the clinician should always observe the patient to make sure that satisfactory eye movements are made. In many commercial devices, the patients' eye movements can be observed through an inbuilt infrared camera, so compliance can be checked on the video monitor. This is desirable, and may be included in future standards. If there is local central or peripheral disease, the fixation points may be easy to see in darkness, but become invisible against a brightly illuminated background, unless the fixation point is surrounded by an (unilluminated) opaque black area. In some patients with very poor vision, it may be necessary to make extreme eye movements if the fixation targets cannot be seen. It has been suggested that in such cases, eye movements of uniform angle can be made with the aid of proprioception: the patient can be seated in a chair with arms, on which the elbows can rest, and the forearms are placed vertically. The patient is encouraged to move his eyes toward the position of one thumb, and then toward the other. It is also possible to have a small buzzer behind each fixation point (with two different tones) to aid the subject in making eye movements of constant amplitude. Finally, if the eye movements displace the electrodes, an artefactual voltage can be recorded. These may occur when passive rotations of the eye are induced to obtain EOGs in small animals or if electrodes on the bridge of the nose are improperly secured. In man, using amplifiers of band pass 0.3–100 Hz alternating saccades give a saw-tooth response. Measurement of the peak-to-peak amplitude of the saw-tooth is easy, and many software packages contain horizontal cursors which can be set, by eye, to pass through the average peak voltage or the average trough voltage. The human visual system is good at making such judgements and disregarding any artefactual voltage or incomplete movements that may occur. All subjects can continue making eye movements for about 10 s, at minute intervals, without fatigue or discomfort. The record derived from such recordings is completely suitable for experimental work. In clinical situations it is common either to make records at 2 min intervals, or to make only a few measurements (for example, with totally blind patients). The aim is to determine the lowest level to which the voltage sinks in darkness (the dark trough) and the peak in subsequent illumination (the light peak). For further details, consult the ISCEV standard (Marmor and Zrenner, 1993).

The magnitude of the ratio of these voltages (often called the Arden ratio) gives an index of the change in RPE voltage which is the end result of the test. However, it is desirable to provide more detail, and various schemes have been proposed to provide standard traces, without the need for measurement and graph drawing. Often records are made on a very slow chart recorder (or its virtual equivalent) so the individual eye movements merge, and the average excursion during the 10 s or so of eye movement during each minute can be visualised as a thick line, and a graph of 20 or more consecutive lines shows the slow change in waveform. In clinical tests, it is important to specify the degree of dark adaptation and the intensity of the subsequent light adaptation because these influence the final ratio obtained. Some workers advocate a prolonged pre-test period during which, in constant dim illumination, the voltage becomes steady. This is desirable, but adds to the duration of the test and is not commonly employed. After this, the subject is put in complete darkness for 12–16 min. This is sufficient time to determine that the voltage has begun to rise from the dark trough level. The time in the dark should be fixed, because, when the light is

turned on, there is a relationship between the dark period and the size of the subsequent rise, although after only 8 min of darkness light rise reaches about 80% of the maximal value obtained with 22 min dark adaptation. The relation between amplitude and time in the dark is approximately exponential (Arden and Kelsey, 1962b).

The light rise response is derived from the entire retina, and therefore a Ganzfeld illumination with "white" light is required. Ganzfeld bowls are recommended, but (especially in view of the eye movements) ad hoc large field viewing (e.g. diffusely and evenly illuminated white walls, floor and ceiling) may suffice. It is important to adjust illumination to compensate for individual variation and individual change in pupil size with illumination. Two alternatives are available: If a very bright light is to be used, then "saturated" responses are obtained, and changes in pupillary diameter compensate for any change in illumination. Alternatively, the pupils may be dilated, and the illumination reduced to obtain a standard retinal illumination. The ISCEV standard recommends 100 cd/m^2 with 7 mm diameter dilated pupils.

Even though much information is available from various manufacturers, it is desirable to establish clinic normal values of the light peak/dark trough ratio. For most centres, the mean value will be 2.2 (220%) with the lower limit of normal ~1.8. This can be strongly affected by small levels of background light in the dark and several labs using total darkness and 15 min dark adaptation report the 5% lower limits of normal as ~2.05. Artifactually, high and low ratios can occur when the whole EOG amplitude is small. The standard therefore requires the report to also say if the absolute sizes of the dark trough and light peak are in normal limits. There is some evidence (see below) that very large values (>3) occur when the TEP is low, but the change in basal conductance is normal: These high values may also indicate an abnormality.

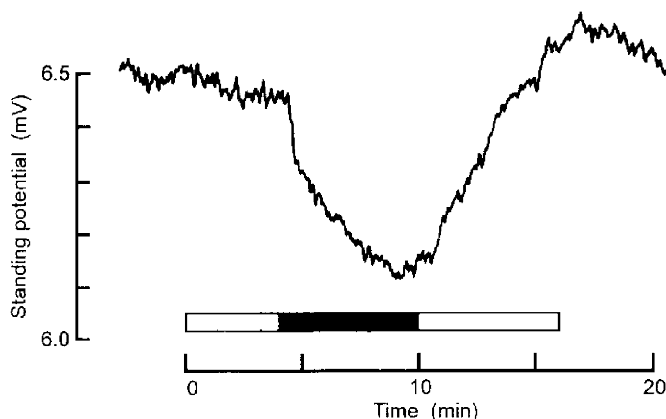

Fig. 12. Sodium bicarbonate (1.4%) infusions in cat causes a decrease in the ocular standing potential. From Segawa et al. (1997). Reprinted with permission of the Japanese Journal of Ophthalmology.

16. Non-photic responses

16.1. Hyperosmolarity, acetazolamide and bicarbonate tests

The light-EOG test depends on the integrity of the retina, the subretinal space and the RPE, and is thus affected by a wide range of pathological processes. The recorded current is generated by large regions of the retina–RPE, and is often normal when localised lesions develop (Gupta and Marmor, 1995). These factors decrease the value of the test. It was hoped that the sensitivity of the test to widespread pathologies could make it useful as a screening test, but other clinical methods (such as fundoscopy, angiography, electroretinography, field testing) provide more detailed information more quickly. A desire to improve the EOG, and obtain a test specific for the RPE has driven workers to investigate non-photic EOGs. In these tests, after a period of recording during which a stable baseline is established, a chemical is infused i.v. It is advisable to set up a venous line before the test begins, and to incorporate a two-way tap in the line so normal saline can be infused during the pre-test period, and the change in infusion made without the subject's knowledge. Otherwise changes of recorded voltage due to anxiety, may affect the test. Recording continues until the decrease in the voltage reaches a lower level. With hyperosmolarity (Kawasaki et al., 1986; Madachi-Yamamoto et al., 1984a) and bicarbonate (Gallemore et al., 1998; Segawa et al., 1997) the infusion causes a reduction in EOG voltage until a trough is reached after 8–10 min (Figs. 12 and 13). With hyperosmolar solutions, a rather slower fall occurs

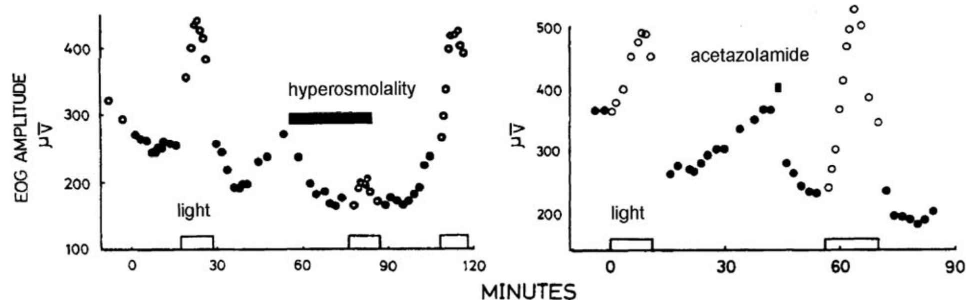

Fig. 13. Hyperosmolality and acetazolamide responses. Note the light rise peak is the same whether acetazolamide has been given or not. Modified from Kawasaki et al. (1977), Madachi-Yamamoto et al. (1984a). Reprinted with permission.

(Gallemore et al., 1998; Madachi-Yamamoto et al., 1984b; Marmor, 1989). Following the trough, the potential may increase slightly if the infusion continues.

The quantity of acetazolamide recommended is 8–10 mg/kg, given by slow intravenous infusion in a period of 1 min and causes a slow decrease in the standing potential. Hyperosmolar solutions used consist of 0.9% normal saline with 25% w/v mannose added, or 15% mannose plus 10% fructose (1400 mOsmolar). The aim is to increase tissue osmolarity by 15–20 mOsmolar. In the bicarbonate test, 0.9% saline, to which sodium bicarbonate, 7% w/v, was added is infused at the rate of 0.83 mg/kg/min. The use of several agents can be combined in a sequence to reduce the number of tests (Gallemore et al., 1998; Gupta and Marmor, 1994, 1995; Leon et al., 1990; Madachi-Yamamoto et al., 1984b; Pinckers et al., 1994).

16.2. Mechanisms and usefulness

During the hyperosmolarity trough the light rise is abolished. However, following administration of acetazolamide the light rise is unaffected so the two tests must provoke different retinal–RPE mechanisms (Gupta and Marmor, 1995; Marmor, 1989). Although experimental work on isolated epithelia has indicated that the hyperpolarisation of the basal membrane may account for the decrease in voltage associated with these agents, the precise mode of action is unknown, so any abnormality detected is merely phenomenological.

There are various reported differences between the light-EOG and the chemically induced changes. Some are of interest in terms of pathology. In Best's disease, the light-EOG is "flat", but the acetazolamide response continues normally (Gupta and Marmor, 1995; Madachi-Yamamoto et al., 1984b; Weleber, 1989) (Fig. 13). Since acetazolamide affects carbonic anhydrase in the RPE, and can enter via the choroidal circulation, this lends some support to the mechanism proposed for the alcohol-EOG (see below). It has been reported (Leon et al., 1990) in a small series of patients with retinal degenerations (particularly X-linked RP) that the response to hyperosmolarity and acetazolamide is more greatly reduced than is the case for the ERG or the light rise. Pinckers et al. (1994) reported that in age-related macular degeneration (ARMD) the acetazolamide response continues normally until choroidal neovascularisation appears. However, in advanced cases of RP, the acetazolamide response is often normal (Gallemore et al., 1998).

Hyperosmolarity EOGs also show abnormality in choroidal neovascular disease but not in ARMD without neovascularisation (Shirao et al., 1997).

17. The alcohol-EOG

In animal experiments (Skoog et al., 1975), and in man (Arden and Wolf, 2000a), a rise in the eye movement voltage occurs after giving alcohol. Unlike the other agents used to produce non-photic EOGs, alcohol can be given by mouth. It also has important differences from the results with the chemically provoked changes described above. Only recently has there been a comparison of the nature and time course of the changes caused by light and the change caused by alcohol. After allowing for the time taken for alcohol to pass from the gullet to the capillaries, both light and alcohol appear to provoke exactly the same complex prolonged voltage changes (within the limit of the experimental precision) (Arden and Wolf, 2000a). Both light and alcohol evoke a rise of voltage to a peak, with a subsequent fall to a trough, and then a second smaller peak. The time for alcohol to travel from the mouth, through the gut and circulation to the eye is minimally 2 min, and the alcohol peaks and troughs are delayed from the light-evoked responses by exactly this interval. It seems that the responses to both agents must be caused in the same manner and by the same mechanisms.

For low doses, the responses of light and alcohol sum. For high doses, light and alcohol "occlude"—i.e. when both agents are given, the response is only very slightly bigger than when each is given alone. These observations imply that the increase in voltage caused by light and by alcohol share a final common pathway. The dose–response curves for the "peak" and "trough" alcohol responses are however quite different (Wolf and Arden, 2005). The latter require ~20 times less alcohol than the peak (the implications are discussed below). The quantity of alcohol required to produce minimal changes in the EOG is very small indeed—about 1 gm infused i.v. over ~20 s or 12 mg/kg taken orally in a 7% v/v solution. When alcohol is taken by mouth in this way, the blood level rises slowly for about 15 min, but the RPE voltage changes are determined by the blood levels in the first few minutes. A standard dose that produces acceptable voltage changes is 226 mg/kg, 20% v/v. With this, the peak blood level never exceeds 80 mg/100 ml and by the end of the test is <40 mg/100 ml, a level widely considered as lower than that which reduces motor efficiency and judgement. The effective minimal blood alcohol concentration causing any voltage change is too small to be measurable with standard tests. This suggests that there is an amplifier between the RPE mechanisms that generate the voltage changes, and the mechanisms that detect the changes in the alcohol composition of the tissues.

Alcohol is known to act on a variety of tissues, including the central nervous system and a number of biochemical pathways have been described (Allansson et al., 2001; Dildy-Mayfield et al., 1992; Ma et al., 2001; Sepúlveda and Mata, 2004). Very few experiments on the physiological effects alcohol have employed such low doses as those that appear effective in the eye.

It is unlikely that CFTR plays a role in the generation of the alcohol-EOG as alcohol does not activate CFTR currents (Marcet et al., 2004). Preliminary findings in our department show that the alcohol-EOG is normal in individuals who are homo- and heterozygous for Δ508 (Fig. 14) (Constable et al., 2005). Furthermore, ethanol

(100 mM) is capable of raising $[Ca^{2+}]_{in}$ in a human RPE cell line (ARPE-19) (Dunn et al., 1996) (Fig. 15) suggesting a direct action of ethanol on the RPE and as such the alcohol-EOG is a sensitive test for RPE function as originally proposed by Arden and Wolf (2000a).

So far, it has been shown that the increase in voltage caused by alcohol response is absent in all cases of RP (Arden and Wolf, 2000b). However the slow fall may persist, even in heterozygotes for X-linked RP (see Fig. 16 (Arden and Wolf, unpublished)). Since ERGs and fundal appearances in such persons are often very nearly normal, and very rarely seriously depressed, the results shown in Fig. 16 may be of significance especially where genetic screens may not be available. It may also have some bearing on pathogenesis because the implication is that the genetic abnormality is expressed in the RPE even in cases where the retinal damage is very small. The alcohol-EOG is also abnormal in ARMD, in a series where the light-EOG test is much less affected (Arden and Wolf, 2003) (Fig. 17).

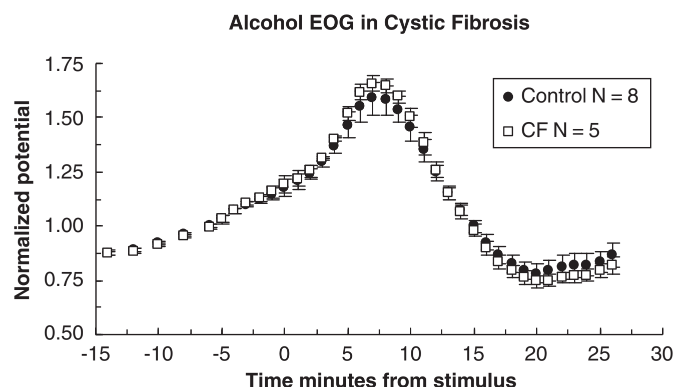

Fig. 14. Preliminary findings a group of individuals with CF ($N = 5$) and controls ($N = 8$) showing normal ethanol responses. Ethanol dose was 110 mg/kg administered orally at $t = 0$. Graphs are mean±SEM. Two were homozygous and three were heterozygous for Δ508. Data normalised to the mean voltage of the 10 min preceding the stimulus. The absolute peak value = 1.59±0.08 for controls and 1.66±0.04 for CF patients. Compare with Fig. 7, where the light-EOGs of the same groups show differences between controls and CF patients.

If, as our preliminary findings suggest, the alcohol-EOG is mediated via a rise in $[Ca^{2+}]_{in}$ then Ca^{2+} homeostasis must be affected in early RP and ARMD and the alcohol-EOG is able to detect such changes before advanced retinal degeneration is apparent. It will be of interest to see in what other cases the alcohol-EOG is also abnormal.

17.1. Relationship between alcohol and light-EOG

The EOG appears to be dose dependent: there is an approximate linear relationship between the logarithm of retinal illumination and the magnitude of the light peak that extends over 3.5 decadic units. More recently, the same has been observed of the dose dependency of the

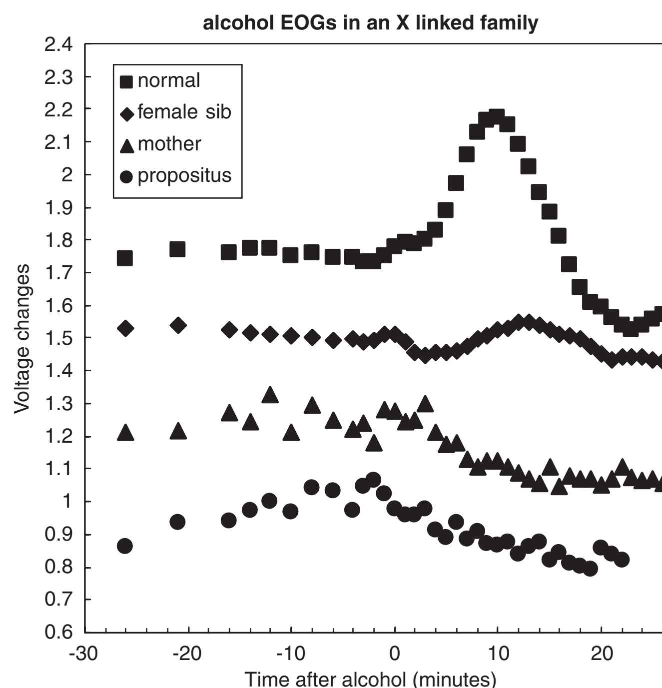

Fig. 16. Alcohol-EOGs in an X-linked family. The propositus is male, and has a severe retinal degeneration. The mother and female sibs are carriers, with normal retinal function, but their EOGs are grossly abnormal. (Arden and Wolf, unpublished).

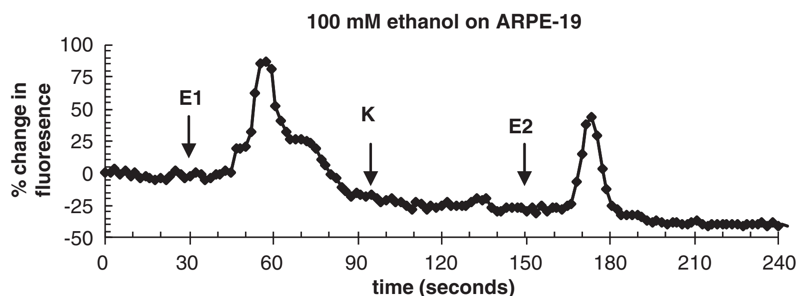

Fig. 15. Response of ARPE-19 cells to 100 mM ethanol loaded with the $[Ca^{2+}]_{in}$ sensitive dye, fluo-4AM. Recordings were made at room temperature in Kreb's solution containing Ca^{2+}. Ethanol 100 mM in Kreb's was added at 30 s (E1) and replaced with Kreb's at 90 s (K) and then challenged again with 100 mM ethanol in Kreb's (E2). The ordinate shows the rise in $[Ca^{2+}]_{in}$ from a single cell expressed as a percentage change in fluorescence from baseline. Note the sharp transient increase and the slower decay.

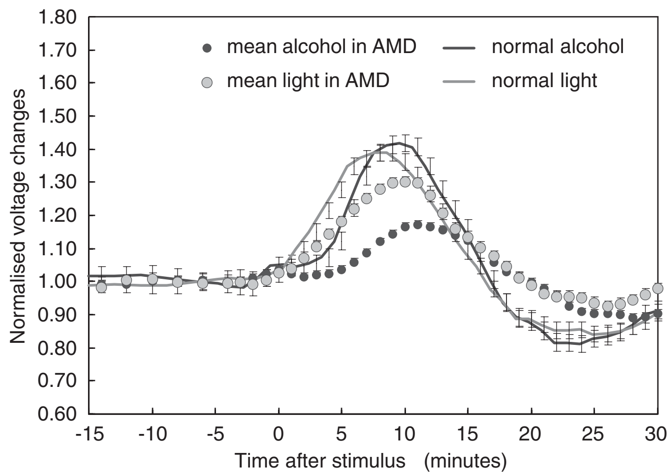

Fig. 17. Comparison of the light- and alcohol-EOGs in normal subjects and patients with ARMD. The standard errors are shown, but they are so low that the loss of the peak response of the light-EOG in patients must be significant, and the additional loss of the alcohol-EOG is also significant. Note that the normalised values are averaged for each minute's records. Compare with Figs. 7 and 14 (from Arden and Wolf, 2003). Reprinted with permission of Investigative Ophthalmology and Visual Science.

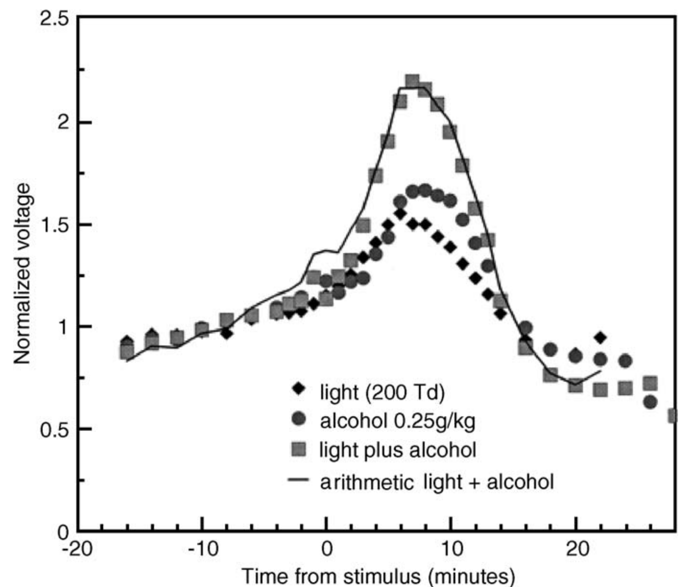

Fig. 18. Alcohol and light: When low light levels and small quantities of alcohol are given, the effects on the EOG are a simple summation (from Arden and Wolf, 2000a). Reprinted with permission of Investigative Ophthalmology and Visual Science.

response to alcohol. It is natural to consider that alcohol may liberate the "light rise substance" but this is not so, because the effects of light and alcohol do not interact as they must do if that were to be the case. Fig. 18 shows that when illumination is increased during the "alcohol rise", the voltage produced by light simply sums with the voltage change produced by alcohol. However, for each agent by itself, there is a pronounced effect of prior stimulation. A prior (low) illumination level suppresses the response to a large step increase of illumination (Fig. 19).

In addition, the presence of a low concentration of alcohol in the blood prevents the development of an "alcohol rise". However, the alcohol response is scarcely affected by illumination, and the light response is scarcely affected by established blood alcohol concentrations. The time course of the production of the "light rise substance" is unknown, but the very similar prolonged responses produced by alcohol can be mimicked by the brief injection of a bolus of alcohol, which rapidly disappears from the circulation. The simplest way of accounting for these findings is that the mechanism that causes the change in potential of the RPE (in this case the depolarisation of the basolateral surface) is a slow intracellular generator within the RPE cells, and a trigger mechanism activates the generator. Extracellular change (of the light substance or alcohol) operates the trigger mechanisms, which then rapidly become desensitised. The triggers release two types of "second messengers" which activate the generator. The implication is that the RPE is responsible for the time course of the EOG, and the voltage–time relationship is not dependent on slow changes in photoreceptors. The work on alcohol has also emphasised the difference between the peak and trough processes in the EOG. The dose–response relationship for peak and trough are different (see above) so that the trough:peak ratio alters systematically with dose (Wolf and Arden, 2004). Moreover, work on the responses of patients with RP show that the positive peak vanishes even in retinas with some preserved rod function, while the fall in voltage remains (Arden and Wolf, 2000b) (Fig. 20). One simple explanation of the fact that positive and negative changes can be separate is that they occur at opposite faces of the RPE. All the agents that provoke non-photic falls in potential might be operating on apical mechanisms, while the alcohol-induced rise, like the light rise seems to occur at the basal surface. A mechanism on the RPE apical surface is apparently a "receptor" for epinephrine, at micromolar concentration (Joseph and Miller, 1992). No such receptor has been demonstrated for alcohol, which is also apparently active at less than micromolar concentration in the RPE. It has been suggested that the alcohol abnormality in ARMD may be the result of a barrier between the RPE and the choroid that prevents entry of alcohol to the sites where it can affect the RPE. This may be of significance in the pathology of ARMD, but has little diagnostic possibility, even though early cases of ARMD show reduction and delay in the alcohol-peak, unless there is a direct and simple relationship between the degree of alcohol-EOG abnormality and the severity of ARMD.

18. Mathematical modelling

In the absence of information about mechanisms, attempts have been made to construct mathematical models that would describe the time course of the EOG changes (Homer and Kolder, 1966; Taumer et al., 1976). These authors treated the changes as a damped oscillation.

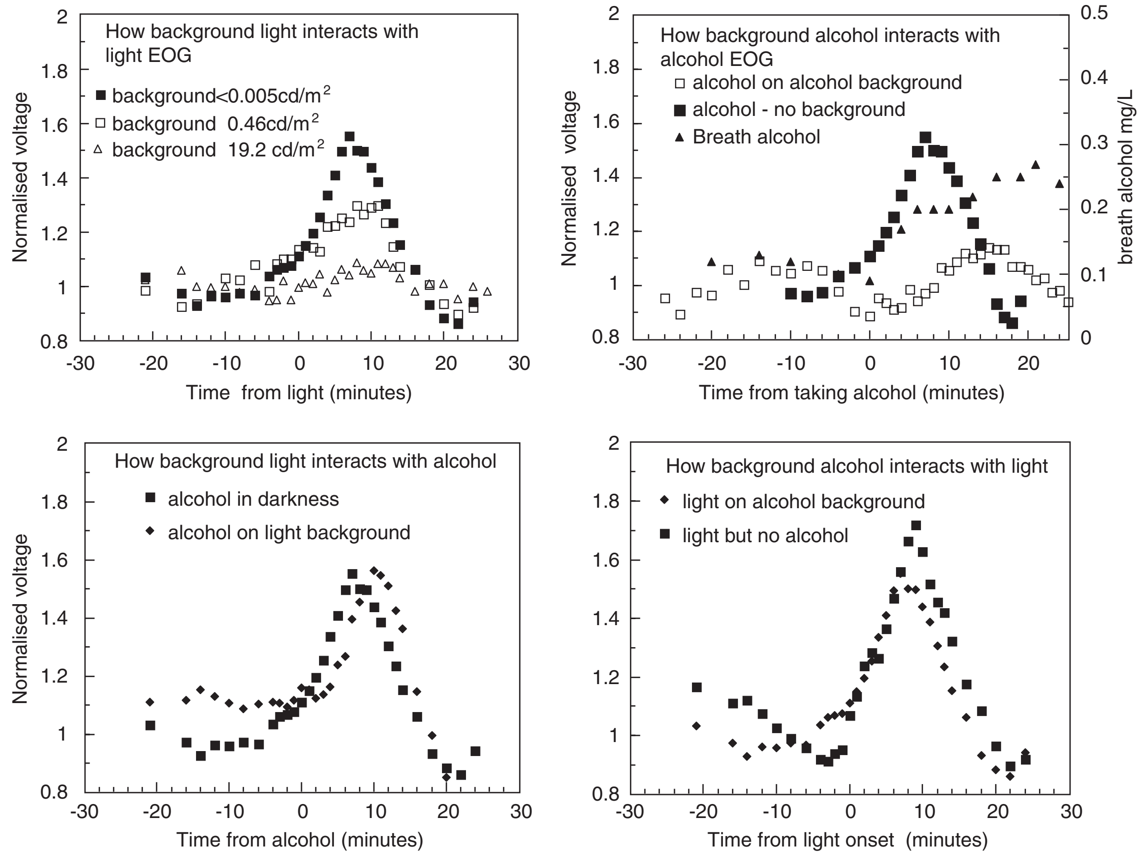

Fig. 19. While backgrounds of low-level light prevent the appearance of a light-induced EOG, and low levels of alcohol inhibit the response to a further dose of alcohol, alcohol has almost no effect on the light rise, and light has almost no effect on the alcohol-induced increase of voltage. The "receptors" for the light rise substance and the receptors (if present) for alcohol must therefore be independent (from Arden and Wolf, 2000a). Reprinted with the permission of Investigative Ophthalmology and Visual Science.

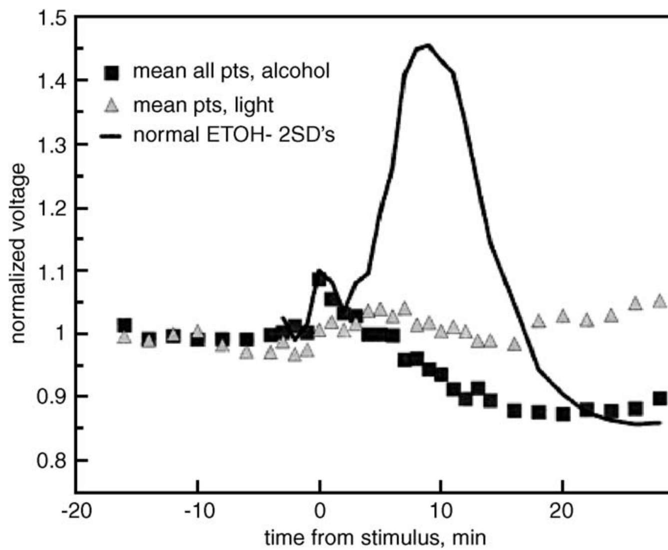

Fig. 20. The average normal alcohol-EOG (full line) is compared to the results in 17 patients with retinitis pigmentosa (squares). The triangles show the same patients' responses to light. It is evident that while the alcohol rise has been lost, a slower fall still occurs. From Arden and Wolf (2000b). Reprinted with permission of Investigative Ophthalmology and Visual Science.

In view of the descriptions above, which indicate that the early rise and the subsequent fall are produced by different mechanisms, it would be expected that the models would be complex, and in fact a number of free parameters have to be introduced before the model predictions correspond to experimental observations—Taumer et al. (1976) indicate that nine free parameters are required to model the EOG.

19. Clinical findings of general interest in the last 10 years

In acute zonal occult outer retinopathy (AZOOR), Francis et al. (2005) used the light-EOG to demonstrate the presence of RPE disturbances and thus help distinguish this condition, commenting "Electrophysiological testing can help avoid lengthy, costly, and potentially invasive investigations" which could be used as a motto by anyone investigating any puzzling defect of vision. The EOG abnormality appears diagnostic in this condition—the only instance (apart from Best's) where this is the case. EOGs are abnormal in cases of Best's associated with mutations of the VMD2 gene, a condition which although genetic have an onset as late as the 4th decade (Renner et al.,

2005)—though see Pollack et al. (2005) and others above for caveats.

Vigabatrin retinopathy has been reported to cause a considerable change to the EOG in many patients, and this is taken as evidence that this drug can alter RPE function (Arndt et al., 1999; Coupland et al., 2001; Harding et al., 2000a, b, 2002; Hardus et al., 2001; Ravindran et al., 2001). The frequency of abnormality is greater than that of the ERG, and is much less in patients on vigabatrin who do not have field defects. If field defects reverse, the EOG abnormality may remain. However, cases have been reported with normal EOGs, and the value of carrying out EOGs in patients under treatment remains undetermined. The antiepileptic drug, lamotrigine has also been associated recently with reduced EOG amplitudes (Arndt et al., 2005).

The prognosis for patients with ARMD treated by a 360° retinotomy and macular translocation is related to the EOG findings. A decreased dark trough is an unfavourable indicator for the return of visual acuity (Luke et al., 2003). Several workers have noticed that in type 1 neurofibromatosis (Lubinski et al., 2001) and in ocular complications associated with interferon alpha (Crochet et al., 2004) a decrease in the absolute value of the EOG voltage indicates damage resulting in loss of vision. In some cases, this may lead to an elevated light–dark ratio. The simplest explanation of this finding is that the absolute voltage depends upon (V_{Basal}–V_{Apical}), and as V_{Apical} is hyperpolarised with respect to V_{Basal}. The change with light depends only on the change in V_{Basal}, and if this is normal the Arden ratio will appear to increase. In conditions where the TEP is reduced but the ratio is normal, it is likely that the recorded voltage is reduced because of a decrease in paracellular resistance. When the absolute value of the voltage is low and the light rise is also reduced, the underlying mechanism maintaining the TEP is likely to be faulty.

It has been reported that malignant melanomata of the posterior uveal tract cause abnormalities in the EOG (Brink et al., 1990), and in a large multicentre study this finding was claimed to be of use in distinguishing between naevi, other forms of retinal detachment, and metastasis into the eye (Spadea et al., 1994, 2002). However, malignant choroidal melanomata have been observed with normal EOGs (McCormick et al., 1996). The EOG has been reported as abnormal in cases of desferrioxamine toxicity (Haimovici et al., 2002; Hidajat et al., 2004), not surprising in view of the effect in reducing the rate of dark adaptation (Arden, 1986). However, other methods of assessing toxicity have been reported (Arden et al., 1984; Davies et al., 1983). A number of authors have emphasised the utility of the EOG in experimental studies involving the genotyping of various retinal degenerations (and especially the carrier states of these conditions), from RP, Usher's syndrome, Best's, helicoidal peripapillary chorioretinal degeneration, neuronal ceroid lipofuscinosis, senior syndrome, choroideremia, butterfly dystrophy and Stargardt's disease (Pojda-Wilczek et al., 2004).

Abnormalities have been reported in multiple evanescent white dot syndrome and in immunodeficiency syndromes (Eysteinsson et al., 1998; Greenstein et al., 2001; Gupta and Marmor, 1995; Harrison and van Heuven, 1999; Jarc-Vidmar et al., 2001; Jurklies et al., 2001; Lafaut et al., 2001; Lim et al., 1999; Miyake et al., 1996; Pinckers et al., 1994, 1996; Ponjavic et al., 1999; Seddon et al., 2001; Stavrou et al., 1996; Theischen et al., 1997; Walter et al., 1994, 1999). In some (e.g. Eysteinsson et al., 1998), it has been reported that in cases of pigmentary disturbance or in degenerations with symptoms occurring delayed to late in life, EOG disturbances are more frequent and profound than are ERG disturbances.

20. Future directions

The identification and roles of the various Cl^- channels in the RPE and how each relates to the generation of the EOG and FOs will become possible when suitable animal models of Best's disease become available. The development of specific mammalian Cl^- channel blockers has already begun to reveal the role of CFTR in the RPE and with further developments the RPE's Cl^- channels' contribution to the EOG components will become clearer. From the clinical point of view, the number of conditions where EOGs have diagnostic value are increasing, and now that it is possible to compare membrane changes evoked (by light) from the retinal surface with those evoked by alcohol acting from the choroidal surface, EOG analysis may serve to produce further information about the pathology of retinal and RPE degenerations.

Acknowledgements

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