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Ion channels and transporters of the retinal pigment epithelium

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ABSTRACT

Ion channels and ion transporters play essential roles in the function of the retinal pigment epithelium (RPE). The use of cell cultures has been exploited as a key method for successfully identifying and studying ion channels and transporters of the RPE. Cultured RPE cells enable robust and long-lasting patch-clamp recordings, Ussing chamber investigations of the transepithelial transport within the isolated RPE, and analyses of the intracellular Ca^{2+} or pH with fluorescent probes. Furthermore, cultured RPE can be transfected at high success rates, permitting the easy use of siRNA to study the involvement of ion channels on the molecular level. However, the expression patterns of the ion channels in the RPE appear to be a very sensitive marker reflecting the extent of RPE differentiation *in vitro*. Having originated from the neuroectoderm, cultured RPE cells seem to retain some capacity to change into a more neuronal phenotype expressing TTX-blockable Na^{+} channels or synaptic Ca^{2+} channels. Therefore, the identification of ion channels and transporters in cultured cells should be verified in freshly isolated RPE cells and *in situ* preparations of the RPE, via immunohistochemistry and the analysis of RPE-specific signals in the electroretinogram from transgenic animals.

1. Ion channels, transporters and RPE function

The retinal pigment epithelium (RPE) is a closely interacting partner of the photoreceptors in the retina and is involved in the visual process (Bok, 1993; Steinberg, 1985; Strauss, 2005). For this purpose, the RPE fulfills multiple functions to maintain photoreceptor activity and structural integrity. A loss of these functions leads to retinal degeneration. Ion channels and ion transporters act to transport ions across the plasma membrane or cytosolic membranes, including those of organelles (Wimmers et al., 2007). The transport of ions, in turn, has a direct or indirect impact on RPE cell function. The direct function is to generate ion fluxes across the plasma membrane for volume regulation, pH regulation or transepithelial ion transport. As an indirect function, ion channels and transporters establish driving forces for ion transport by determining the transmembrane potentials or by regulating cell function using intracellular free Ca^{2+} as a second messenger. The following review summarizes techniques and conditions for a successful analysis of the ion channels and transporters using cultured RPE cells. Examples of a successful analysis will be given. These examples concentrate on the data from cultured cells and might therefore be incomplete regarding the full physiological mechanisms or the types of ion channels/transporters expressed in RPE in its native environment in the intact retina.

2. The use of cell culture to study ion channels and transporters in the RPE

2.1. The methods used to culture the RPE cells for ion channel/transporter analysis

A large variety of ion channel or ion transporter properties can only be studied in cell culture. Cell culture offers the advantage of analyzing isolated single RPE cells or isolated RPE monolayers independently from the neighboring tissues. This is a requirement for most of the methods that enable the direct investigation of ion channels: patch-clamp analysis, Ussing chamber recordings and the measurements of the cytosolic free Ca^{2+} with fluorescent dyes as probes. Primary RPE cell cultures or cell lines may both be used for ion channel/transporter studies. RPE cell lines exist for a variety of species, including humans, rats or mice. Primary RPE cell cultures can be established from both fetal and adult RPE cells. The fetal cells display the highest potential for modulation towards a fully characterized RPE phenotype and can be therefore driven to a very high level of differentiation (Hu and Bok, 2010; Maminishkis et al., 2006). Important markers for this differentiation are the expression of bestrophin-1, RPE65 and ZO1. In primary cultures, the

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expression of both bestrophin-1 or of RPE65 decreases very quickly after establishing the culture. The expression of ZO1 suggests the formation of a polarized RPE monolayer which, however, is not sufficient because ZO1 belts can be incomplete. The final proof would be the development of a representative transepithelial resistance. The highest degree of polarization can be achieved when the cells are grown on permeable filter supports (Burke, 2008). Cultivation on permeable substrates serves to support features such as transepithelial resistance and potential, at levels comparable to those observed in fresh preparations of intact RPE tissue. On permeable filter supports, the cultured RPE cells can be studied in modified Ussing chambers (see below). RPE cells used for fluorescence measurements are most conveniently cultured on glass cover slips, enabling their mounting on stages of various designs on inverted microscopes. For the patch-clamp analysis, only subconfluent cultures should be used, because more sparsely plated cells enable stable patch-clamp configurations for longer recording periods and are more suitable for patch-clamp because of reduced extracellular matrix. We have recently developed a method to use adult RPE cultures whose properties are very close to those of freshly isolated cells. In this method, primary cultured porcine RPE cells are seeded at a density approaching that of native tissue, on either glass cover slips or permeable filter supports. These cultures are used for 72 h maximum and are not subcultured. Using this method, the RPE cells form a virtually confluent monolayer without proliferation, and conserve bestrophin-1 expression for more than 72 h. This short term culture is easily accessible for siRNA treatment and can be used for fluorescence-based methods.

2.2. Patch-clamp analysis

The patch-clamp technique permits the direct, real time characterization of ion channels, either in the single-channel mode or the whole-cell mode (Hamill et al., 1981; Sakmann and Neher, 1984). In the single channel mode, the ion channel's properties can be investigated at the molecular level. In the whole-cell configuration, currents through all of the active ion channels in the RPE plasma membrane can be measured at the same time. Most of the studies on ion channels in the RPE have been performed in the whole-cell mode. The reason for this is that the absence of an ion channel in the single-channel analysis does not justify the conclusion that this ion channel is generally not expressed by RPE cells. Because the cells of the retinal pigment epithelium are electrically coupled to each other (Himpens and Vereecke, 2000), the measurement of the ion channels in the plasma membrane by the whole-cell patch-clamp technique requires isolated single cells. In confluent cells ionic currents not only can flow across the cell membrane but also spread among neighboring cells through gap-junction channels. In this case the patch-clamp amplifier is not able to clamp the membrane potential properly. Although whole-cell recordings from freshly isolated RPE cells have been published, the success rate and durability of whole-cell recordings with cultured cells is much higher. This is a prerequisite for experiments that aim to analyze ion channel regulation to provide further information on their role in RPE function. A variety of ion transporters, such as the Na^+/Ca^{2+}-exchanger, can also be studied using this technique (Grewer et al., 2013), although it was not used for the RPE cells.

2.3. Ussing chamber recordings

The Ussing chamber was developed by Ussing to study ion transport across epithelia (Ussing, 1949, 1953, 1972). The Ussing chamber allows the investigation of ion transporters and ion channels in the intact epithelial tissue or sheet, thus identifying their apical to basolateral polar distribution. The basic principle of the Ussing chamber is reflected by its two independently bathed halves, which are separated by the epithelium. Inside each chamber, electrodes are placed in close proximity to the epithelium to record the electrical potentials that result from the transepithelial ion transport by the epithelium. To obtain a measure of the transepithelial ionic current, which is produced by the active ion transport activity of the epithelium, an additional two current electrodes are placed inside each chamber at a greater distance from the epithelial surfaces, to generate a homogeneous electrical field distributed over the epithelium. With these electrodes, the so-called short-circuit current is measured: the current required to adjust the transepithelial potential to zero is a direct measure of the transepithelial ionic current generated by the epithelium to establish the transepithelial potential. As a third important epithelial transport parameter, transepithelial resistance is measured as change in voltage proportional to small current injections. The transepithelial resistance results from both the para- and trans-cellular resistances to ion flow. When ion transport across the epithelium is stimulated and when the ion conductance of either the apical or the basolateral membrane is increased, a decrease in the transepithelial resistance can be observed. To identify the specific nature of the transported ion and its transport direction, the present Ussing chamber measurements can be combined with a flux analysis of specific radioactive tracer ions. Ion transporters or ion channels and their apical to basolateral distributions can be easily analyzed in the Ussing chamber because the two halves of the chamber are perfused separately. In this way, a blocker for an ion channel or transporter can be applied preferentially to the apical or the basolateral side. Using a rather large specimen of the transporting epithelia, such as those from frog skin or gut, these kinds of studies have been routinely performed using freshly isolated tissue. To study transport phenomena across the freshly isolated RPE, much work has been performed in RPE-Bruch's membrane-choroid preparations, which combine Ussing chamber with microelectrode recordings; the latter can also measure transport across either the isolated apical or basolateral membrane (Hughes et al., 1998; la Cour, 1985; Steinberg, 1985). In addition, RPE monolayers can be easily cultured on permeable filter supports and mounted onto specially designed Ussing chambers, revealing multiple mechanisms of regulating transepithelial ion transport (Arndt et al., 2001; Defoe et al., 1994; Hernandez et al., 1995; Hu et al., 1996, 1994; Loewen et al., 2003; To and Hodson, 1998). Depending on the cell culture technique, these cultured RPE cells show basic transepithelial parameters, such as transepithelial resistance of 170–400 $\Omega\ cm^2$ (e.g. Ammar et al., 1998; Hernandez et al., 1995; Hu et al., 1994, 1996), which under appropriate conditions fall within the range of 300–1600 $\Omega\ cm^2$ depending on species and preparation (e.g. Arndt et al., 2001; Bialek et al., 1995; Blaug et al., 2003; Gallemore et al., 1993; Joseph and Miller, 1991; Kuntz et al., 1994; Peterson et al., 1997) characterized by freshly isolated tissues using combined Ussing chamber and microelectrode recording techniques. Recently improved cell culture techniques from human fetal cells resulted in RPE monolayers with transepithelial resistances between 500 and 800 $\Omega\ cm^2$ (Hu and Bok, 2010; Maminishkis et al., 2006).

2.4. Ca^{2+} imaging

Many critical functions of the RPE are regulated by changes in the concentration of intracellular free Ca^{2+}, which may act as a second messenger; such signals are often generated by the activation of Ca^{2+}-conducting ion channels. Ca^{2+} imaging not only allows the identification of ion channels contributing to Ca^{2+} signals but also of the signaling pathways regulating RPE cell's functions, including secretion or proliferation. The method is based on Ca^{2+}-