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DISTRIBUTION AND FUNCTION OF POLYCYSTIN-2 IN MOUSE RETINAL GANGLION CELLS

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Abstract—The polycystin family of transient receptor potential (TRP) channels form Ca^{2+} regulated cation channels with distinct subcellullar localizations and functions. As part of heteromultimeric channels and multi-protein complexes, polycystins control intracellular Ca^{2+} signals and more generally the translation of extracellular signals and stimuli to intracellular responses. Polycystin-2 channels have been cloned from retina, but their distribution and function in retinal ganglion cells (RGCs) have not yet been established. In the present study, we determined cellular and subcellular localization as well as functional properties of polycystin-2 channels in RGCs. Polycystin-2 expression and distribution in RGCs was assessed by immunohistochemistry on vertical cryostat section of mouse retina as well as primary cultured mouse RGCs, using fluorescence microscopy. Biophysical and pharmacological properties of polycystin-2 channels isolated from primary cultured RGCs were determined using planar lipid bilayer electrophysiology. We detected polycystin-2 immunoreactivity both in the ganglion cell layer as well as in primary cultured RGCs. Subcellular analysis revealed strong cytosolic localization pattern of polycystin-2. Polycystin-2 channel current was Ca^{2+} activated, had a maximum slope conductance of 114 pS, and could be blocked in a dose-dependent manner by increasing concentrations of Mg^{2+}. The cytosolic localization of polycystin-2 in RGCs is in accordance with its function as intracellular Ca^{2+} release channel. We conclude that polycystin-2 forms functional channels in RGCs, of which biophysical and pharmacological properties are similar to polycystin-2 channels reported for other tissues and organisms. Our data suggest a potential role for polycystin-2 in RGC Ca^{2+} signaling.

Key words: polycystin, transient receptor potential channel, retinal ganglion cells, calcium, electrophysiology.

Calcium homeostasis and signaling are tightly regulated in excitable cells (Koulen and Thrower, 2001; Berridge et al., 2003), including neurons of the retina (Akopian and Witkovsky, 2002), and control a large number of intracellular mechanisms, such as gene expression, neurotransmitter and hormone secretion, and apoptosis (Koulen and Thrower, 2001; Berridge et al., 2003; Duncan et al., 2010) through modulation of the intracellular free Ca^{2+} concentration.

Two pathways exist that permit Ca^{2+} entering the intracellular milieu: (1) from the extracellular space through voltage-, ligand-, store-, or second messenger-activated Ca^{2+} channels (Berridge et al., 2003) and (2) from intracellular stores such as the endoplasmic reticulum (ER) (Berridge et al., 2003). Besides Ca^{2+} entering the cell through these pathways, Ca^{2+} uptake into intracellular stores or Ca^{2+} extrusion to the extracellular space are under cellular control and critically shape cellular Ca^{2+} transients and Ca^{2+} homeostasis (Koulen and Thrower, 2001; Berridge et al., 2003), however, their mechanisms are less well understood.

Three classes of intracellular Ca^{2+} channels exist, each containing several isoforms, each of which display temporospatially distinct expression patterns, biophysical properties, and physiological functions (Koulen and Thrower, 2001): inositol-1,4,5-trisphosphate receptors (IP_3Rs), ryanodine receptors (RyRs), and polycystin-2 family transient receptor potential channels (TRPPs). The group of TRPPs comprises eight proteins, three of which have been shown to be cation channels (for review see Gees et al., 2010): polycystin-2, polycystin-L, and polycystin-2L2.

The mammalian retina contains a large number of specialized cell types (Masland, 2001a,b). Retinal ganglion cells (RGCs) convert the synaptic input into spike output that transduces visual information to the brain for processing, and hence, are critical for visual function (Nassi and Callaway, 2009). Recent progress has been made in elucidating the signaling pathways in RGC dendrites involving voltage-gated Ca^{2+} channels (Margolis et al., 2010), however, surprisingly little is known about expression, distribution, and function of intracellular Ca^{2+} channels in RGCs. We previously reported the differential distribution of IP_3Rs in RGCs (Mafe et al., 2006), and polycystin-L and polycystin-2L2 were detected in retina (Nomura et al., 1998; Wu et al., 1998; Guo et al., 2000). The lack of knowledge on the role of polycystin-2 in the retina is surprising, given the tentative association between polycystic kidney disease and macular defects in patients and experimental models (Narendran et al., 2004; Feng et al., 2009).

A better understanding of the expression, distribution, and function of polycystin-2 will provide the basis for further elucidation of the intracellular Ca^{2+} signaling pathways in RGCs and their putative role in pathologies such

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Abbreviations: BLM, bilayer lipid membrane; Brn3a, brain-specific homeobox/POU domain protein 3A; BSA, bovine serum albumin; CICR, Ca^{2+}-induced Ca^{2+} release; DAPI, 4′,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; IP_3Rs, inositol-1,4,5-trisphosphate receptors; NFM, neurofilament; PBS, phosphate buffered saline; RGCs, retinal ganglion cells; RyRs, ryanodine receptors.

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as glaucoma, in which loss of RGCs can ultimately lead to blindness (Fan and Wiggs, 2010).

Here, we report the subcellular distribution, expression, and functional characterization of polycystin-2 channels in mouse RGCs.

EXPERIMENTAL PROCEDURES

Animals

Male adult albino Swiss-Webster and C57/B6 mice (4–6 weeks of age; The Jackson Laboratory, Bar Harbor, ME, USA) were euthanized by CO_2 overexposure. All experiments described herein were in compliance with the guidelines for the welfare of experimental animals issued by the National Institutes of Health, in accordance with institutional guidelines and the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. The authors attest that all efforts were made to minimize the number of animals and their suffering.

Materials and antibodies

Cell culture reagents were obtained from Gibco® (Invitrogen, Carlsbad, CA, USA). Unless otherwise indicated, all other reagents were obtained from Sigma (St. Louis, MO, USA).

The mouse anti-CD90 (Thy1.2) was obtained from Caltag (Burlingame, CA, USA) and used at 1:200 for immunohistochemistry and immunocytochemistry. Rabbit anti-neurofilament (NFM) 68 kDA (1:1000 for immunocytochemistry), mouse anti-OX42 (IgG2a; 1:200), mouse anti-Brn3a (clone 5A3.2; 1:200 for immunocytochemistry), and rabbit anti-PC-2 (#AB9088; 1:1000 for immunohistochemistry and immunocytochemistry) were purchased from Millipore (Temecula, CA, USA).

Fluorescently conjugated secondary antibodies (AlexaFluor® 488 goat anti-rabbit IgG and AlexaFluor® 594 goat anti-mouse IgG; Invitrogen, Carlsbad, CA, USA) were used at 1:2000. ProLong® Antifade (Invitrogen) was used for mounting and nuclear staining of cultured RGCs. 4′,6-Diamidino-2-Phenylindole (DAPI; EMD Chemicals, Gibbstown, NJ, USA; 1:50,000 dilution) and Aqua-Poly/Mount (Polysciences Inc., Warrington, PA, USA) were used for nuclear staining and mounting of retina sections.

Preparation of retinal cultures

RGCs were prepared from 4 to 6 weeks old male albino Swiss Webster mice (six retinae from three different animals per preparation) and cultured as described by us previously (Mafe et al., 2006). Briefly, eyes were enucleated and dissected in Neurobasal A medium (Invitrogen). Retinae were enzymatically dissociated by incubation in papain solution (34 U/ml papain, 3.3 mM DL-cysteine, 0.4 mg/ml bovine serum albumin in Neurobasal A medium for 25 min at 37 °C), washing three times in culture medium, and mechanical trituration. Cells were plated onto 12 mm glass coverslips coated with poly-D-lysin/laminin (BD Biocoat™, BD Biosciences, Bedford, MA, USA) and maintained for 14 days in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were grown in culture medium (Neurobasal A supplemented with 1×B27, 100 U/ml penicillin, 100 μg/ml streptomycin, 110 mg/ml pyruvate, 292 ng/ml glutamine, 1% fetal bovine serum, 5 ng/ml insulin, 100 μg/ml transferrin, 100 μg/ml crystalline bovine serum albumin (BSA), 60 ng/ml progesterone, 16 μg/ml putrescine, 40 ng/ml sodium selenite, 40 ng/ml thyroxine, 40 ng/ml tri-iodothyronin, 50 ng/ml brain derived neurotrophic factor, 10 ng/ml ciliary neurotrophic factor, 2 mg/ml forskolin, and 10 ng/ml fibroblast growth factor b).

Immunocytochemistry

Immunocytochemistry was carried out as described by us previously for RGCs (Mafe et al., 2006). Briefly, after 7–14 days of culture, RGCs were fixed using 4% paraformaldehyde in 10 mM phosphate buffered saline pH 7.4 (PBS) for 30 min. Cells were permeabilized in 3% normal goat serum (NGS), 1% BSA, and 0.05% Triton X-100 following a 1 h block in PBS containing 10% NGS, 1% BSA, and 0.05% Triton X-100. Primary antibody was applied and coverslips incubated overnight at 4 °C in a humidified chamber, protected from light. Following wash with PBS, secondary antibody was applied for 1 h at RT in a humidified chamber and protected from light. After washing, coverslips were mounted on slides using ProLong® Antifade (Invitrogen, Carlsbad, CA, USA). Negative controls consisted of the omission of either primary or secondary antibody from the incubation steps and were performed at the same time (data not shown). Immunofluorescence labeling was examined and recorded using an Olympus IX70 microscope (Olympus Corp., Center Valley, PA, USA). We performed immunocytochemistry on four separate cultures of RGCs (n=4).

Immunohistochemistry

Immunohistochemistry was carried out essentially as previously described (Koulen and Brandstätter, 2002; Kaja et al., 2003; Koulen et al., 2005a; Mafe et al., 2006). Eye cups were immersion fixed in 4% paraformaldehyde in PBS for 20 min. Tissue was cryoprotected by infusion with 30% sucrose and 0.05% sodium azide in PBS. Vertical cryosections (12 μm) were cut on a cryostat (Leica Microsystems, Bannockburn, IL, USA). Sections were collected on silane coated slides (Mercedes Medical, Sarasota, FL, USA) and dried for 30 min at RT. Immunofluorescence labeling was examined, recorded, and analyzed using a Leica SP5X white light laser, laser scanning microscope (Leica Microsystems, Bannockburn, IL, USA), and the Leica Application Suite Advanced Fluorescence Software v2.4.1 (Leica Microsystems). Negative controls consisted of the omission of either primary or secondary antibody from the incubation steps and were performed at the same time (data not shown). Furthermore, specificity of the antibody was confirmed by pre-incubation with 100-fold excess of the peptide used for antibody generation (#AG565, Millipore, Temecula, CA, USA). Immunoreactivity was measured in three sections each for a total of four eyes (each obtained from a different mouse).

Single channel electrophysiology

ER membrane-enriched vesicles from immunopanned RGCs (obtained from retinae of 12 animals) were used in planar bilayer lipid membrane (BLM) electrophysiology as described previously (Koulen and Ehrlich, 2000; Koulen et al., 2001, 2002, 2005a,b; Westhoff et al., 2003; Hayrapetyan et al., 2008; Rybalchenko et al., 2008, 2009; Kaja et al., 2011b) using a Warner Instruments BLM workstation (Warner Instruments, LLC, Hamden, CT, USA).

Single channel activity was determined with BLM cis solution corresponding to the cytosol on the cytosolic side of the channel [250 mM HEPES-Tris, pH 7.35] and BLM trans-solution corresponding to the ER lumen on the ER luminal side of the channel [250 mM HEPES, 55 mM $Ba(OH)_2$, pH 7.35] under voltage-clamp conditions. Other cationic conductances were measured by substituting the appropriate hydroxide salt for $Ba(OH)_2$. Ryanodine receptor activity was employed to identify the proper orientation during incorporation of ER membrane proteins into the BLM (Koulen et al., 2002). Purity of the endoplasmic reticulum membrane preparation was determined using standard markers of cellular sub-compartments, calnexin, sodium/hydrogen exchanger 3, epidermal growth factor receptor, and the Golgi 58K protein, as described previously (Koulen et al., 2002). Addition to the cis-side of the BLM, that is, the cytosolic side of the ER, was used for pharmacological agents and physiological modulators. Addition to