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Research paper

Extramitochondrial tricarboxylic acid cycle in retinal rod outer segments

Isabella Panfoli [a,*], Daniela Calzia [a], Silvia Ravera [a], Maurizio Bruschi [b], Carlo Tacchetti [c], Simona Candiani [a], Alessandro Morelli [a], Giovanni Candiano [b]

[a] Department of Biology, University of Genoa, Viale Benedetto XV, 5, 16132 Genova, Italy
[b] Laboratory on Pathophysiology of Uraemia, G. Gaslini Institute, Via V Maggio 39, 16148 Genova, Italy
[c] IFOM Center for Cell Oncology and Ultrastructure, Department of Experimental Medicine, University of Genoa, Via de Toni 14, 16132 Genoa, Italy

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ABSTRACT

Vertebrate retinal rod Outer Segments (OS) are the site of visual transduction, an energy demanding process for which mechanisms of ATP supply are still poorly known. Glycolysis or diffusion of either ATP or phosphocreatine from the Inner Segment (IS) does not seem to display adequate timing to supply ATP for phototransduction. We have previously reported data suggesting an aerobic metabolism in OS, which would largely account for the light-stimulated ATP need of the photoreceptor.

Here, by oxymetry and biochemical analyses we show that: (i) disks isolated by Ficoll flotation consume O_2 in the presence of physiological respiring substrates either in coupled or uncoupled conditions; (ii) OS homogenates contain the whole biochemical machinery for the degradation of glucose, i.e. glycolysis and the tricarboxylic acid cycle (TCA cycle), consistently with the results of our previous proteomic study. Activities of the 8 TCA cycle enzymes in OS were comparable to those in retinal mitochondria-enriched fractions. Disk and OS preparations were subjected to TEM analysis, and while they can be considered free of inner segment contaminants, immunogold with specific antibodies demonstrate the expression therein of both the visual pigment rhodopsin and F_oF_1-ATP synthase. Finally, double immunofluorescence on mouse retina sections demonstrated a colocalization of some respiratory complex mitochondrial proteins with rhodopsin in rod OS.

Data, suggestive of the exportability of the mitochondrial machinery for aerobic metabolism, may shed light on those retinal pathologies related to energy supply impairment in OS and to mutations in TCA enzymes.

1. Introduction

Visual phototransduction converts light into neuronal signal in rod and cone cells of the vertebrate retina. The rod Outer Segments (rod OS), which is devoid of mitochondria, is a specialized compartment of the retinal photoreceptor containing a stack of membranous disks surrounded by plasma membrane, that house the integral and peripheral membrane proteins performing photon capture and visual transduction [1]. Rod OS are continuously renewed. Disks are synthesized at the base of the rod, move toward the OS tip, where the upper disks are phagocytized by the retinal pigment epithelium (RPE) [2].

Phototransduction and visual recovery processes in rod OS require a considerable amount of energy for the turnover of nucleotides including ATP, GTP, NAD^+ and $NADP^+$. During the excitation phase of the vertebrate photoresponse, a light-stimulated enzymatic cascade culminates in the hydrolysis of cyclic GMP (cGMP) [1,3]. The fall in cGMP concentration generates the neuronal signal. The recovery of photoresponse requires synthesis of cGMP from GTP by a guanylyl cyclase stimulated by the lowered Ca^{2+} levels following photoexcitation [1]. However, the source of GTP, i.e. of ATP, in OS is still puzzling [4].

In early studies, the pentose cycle was described in rod OS [5], and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was found associated with the bovine OS plasma membrane [6]. Six enzyme activities of the glycolytic pathways were assayed in purified rod OS preparations [7,8].

The general view was that the energy for phototransduction is provided by the inner segments, where all the mitochondria of the

Abbreviations: ACL, ATP-citrate lyase; ETC, electron transport chain; IS, rod inner segment; OS, rod outer segment; OXPHOS, oxidative phosphorylation; TCA cycle, tricarboxylic acid cycle.

* Corresponding author. Viale Benedetto XV, 3, 16132 Genova, Italy. Tel.: +39 010 353 7397; fax: +39 010 353 8153.
E-mail addresses: Isabella.Panfoli@unige.it (I. Panfoli), dcalzia@gmail.com (D. Calzia), silvia.ravera@gmail.com (S. Ravera), mauriziobruschi@yahoo.it (M. Bruschi), Carlo.Tacchetti@unige.it (C. Tacchetti), candiani@unige.it (S. Candiani), morellia@unige.it (A. Morelli), giovannicandiano@ospedale-gaslini.ge.it (G. Candiano).

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photoreceptor cell are contained. However, it was shown that anaerobic glycolysis in the OS does not have the capacity to satisfy the higher energy demand following photoexcitation, as illumination increases cGMP turnover by 5-folds exceeding the glycolytic capacity of rod OS to produce ATP [4,9–11]. Also, the hypothesis that a creatine phosphate pool is supplied to the rod OS from the inner segment (IS) by a phosphocreatine shuttle pathway [9,12] seems unlikely, as recently it was shown that only the apical disks are functional in phototransduction due to the progressive loss of cholesterol, which inhibits Rh [13]. Therefore the timing of the process (minutes) [14] would not fit to that of photoreception at the apical disks, set at milliseconds [1]. It may seem that our overall understanding of ATP supply in OS is inadequate. However, our results [15] on bovine rod OS disks cogently suggest that these express functional mitochondrial respiratory chains. A consistent ATP synthesis, dependent on a transmembrane electrochemical gradient of protons, and a respiratory activity were measured [15]. An extracellular ATP synthesis in the nanomolar range was also described on the surface of hepathomes [16] and of the human umbilical endothelial cells [17]. Electron Transfer Chain (ETC) complexes activity was also measured and microscopy techniques identified mitochondrial proteins in the disks [15]. It is tempting to presume that the additional energy required for cGMP regeneration during visual recovery comes from an extramitochondrial aerobic metabolism [15].

Glycolysis harvests but a fraction of the ATP available from glucose consumption. The aerobic processing of glucose with the complete oxidation of the acetyl group of Acetyl CoA to carbon dioxide (CO_2) and water is the source of a considerable amount of ATP. This oxidation takes place in the Tricarboxylic Acid (TCA) Cycle, the final common pathway for the oxidation of fuel molecules [18]. Our proteomic analysis of disk proteins showed the expression of many mitochondrial proteins, among which most of the proteins of the ETC and many TCA cycle enzymes [19]. A proteomic analysis of rod OS was recently reported [20], focused on proteins implicated in vesicle trafficking and membrane fusion.

Here we show that, besides NADH already reported [15], Pyr/Malate are respiring substrates for isolated disks. Consistently with this, and with our previous proteomic analysis of disks [19], we show that the 8 enzymes of the TCA cycle, the gateway to the aerobic metabolism, are catalytically active in OS and their activity is comparable to that found in retinal mitochondria-enriched fractions. All of the glycolytic enzymes were also assayed and found active in OS.

Results are discussed in terms of the implications of the existence of a respiration in OS in many pathological conditions affecting rods.

2. Materials and methods

2.1. Materials

Ficoll, protease inhibitor cocktail, leupeptin, Ampicillin, salts, respiratory chain inhibitors (Rotenone, Antimycin A), Nigericin, Valinomycin, rabbit anti-ATP synthase beta subunit and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water (Milli-Q; Millipore, Billerica, MA, USA) was used throughout. All reagents were of analytical grade.

2.2. Sample preparations

2.2.1. Purified bovine rod OS preparations

Retinas were extracted by a procedure that maximizes rod OS yield: retinas were let to float in after incubating them in the eye semicup free of vitreous and lens, in Mammalian Ringer (MR): 0.157 M NaCl, 5 mM KCl, 7 mM Na_2HPO_4, 8 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$ pH 6.9 plus protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA) and 50 μg/ml Ampicillin) for 10 min. Then retinas are cut free of the optic nerve with a scissor. Purified bovine rod OS were prepared under dim red light from 14 retinas (from freshly enucleated bovine eyes, obtained from a local slaughterhouse) at 4 °C, by sucrose/Ficoll continuous gradient centrifugation [19,21] in the presence of protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO) and ampicillin (100 μg/ml). This method isolates OS with an intact plasma membrane. Rod OS preparations were routinely characterized for integrity of plasma membrane as reported in Schnetkamp [22]. OS homogenates were obtained by Potter–Elveheim homogenization on ice in 1:1 (w/v) hypotonic medium (5 mM Tris–HCl, ph 7.4 plus protease inhibitor cocktail and ampicillin).

2.2.2. Osmotically intact disk preparations

Osmotically intact disks were prepared by Ficoll flotation from purified rod OS [23]. OS were allowed to burst for 1 h in distilled water with 70 μg/ml leupeptin, and Ampicillin (100 μg/ml) at 4 °C. Then suspension was layered onto 5% Ficoll solution in distilled water with 70 μg/ml leupeptin, and Ampicillin (100 μg/ml) and centrifuged for 2 h at 25,000 rpm in a Beckman FW-27 rotor (120,000 *g*). Osmotically intact disks were collected at the interface between water and Ficoll under sterile conditions. A_{280}/A_{500} ratio was 1.8 ± 0.1 (average ± SD) and Rh concentration was 0.8 mM [19].

OS and disks were prepared in the absence of Cyclosporin A and 2-Aminoethoxydiphenyl borate [24], inhibitors of the mitochondrial permeability transition pore (MTP) opening [25]. Inhibitors of the MTP were not employed as they proved not necessary, in fact respiration was observed in their absence. Furthermore, these conditions would promote the MTP formation in contaminant mitochondria, if any, during the disk isolation procedure, so these would not be functional.

2.2.3. Retinal mitochondria-enriched preparations

All steps were performed at 4 °C. Bovine retinal mitochondria-enriched fractions were isolated by standard differential centrifugation techniques from residual retinas after rod OS preparation. Residual retinas (4 g) were resuspended in 4 ml of 0.32 M Sucrose, 5 mM N-2-hydroxyethylpiperazine-N1-2-ethanesulfonic acid (HEPES) pH 7.2, protease inhibitor cocktail, 30 nM Cyclosporin A, 30 nM 2-Aminoethoxydiphenyl borate and 0.060 mg/ml Ampicillin, vortexed for 30 s and centrifuged at 700 g for 10 min, in Heraeus centrifuge. Pellet was discarded, supernatant centrifuged at 10,900 *g* for 10 min. Pellet was retained as a mitochondria-enriched preparation, stored on ice and used within 4 h of isolation. Respiratory control ratio values of these preparations were 4.7 ± 0.2 with Pyr/Malate as substrates.

2.3. TEM microscopy

Disk and OS samples, fixed in 3% paraformaldehyde and glutaraldehyde 0.2% were included in gelatin and frozen in liquid nitrogen. Ultrathin sections (20 nm thick) obtained with a microtome were placed on copper grids (1 × 1 mm). TEM experiments were performed on a Tecnai 12-G2 EM (FEI Company), equipped with compustage. Sections were labelled with anti-Rh (1:200) or anti-ATP synthase beta subunit (1:100) as primary Ab. Rh primary Ab was recognized by rabbit anti-mouse secondary Ab and protein-A bound to colloidal gold (15 nm) (Amersham Biosciences, Piscataway, NJ). For F_oF_1-ATP synthase primary Ab was recognized by anti 15 nm gold-labeled goat anti-rabbit IgG, 1:50 (ICN). All steps were at room temperature.