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A variant of arrestin-1 binds rod outer segment membranes in a light-independent manner

Graciela L. Uzcanga [a,1], Aniuska R. Becerra [b,2], Deisy Perdomo [a], José Bubis [a,*]

[a] *Departamento de Biología Celular, División de Ciencias Biológicas, Universidad Simón Bolívar, Apartado 89.000, Valle de Sartenejas, Baruta, Caracas 1081-A, Venezuela*
[b] *Departamento de Química, Universidad Simón Bolívar, Apartado 89.000, Valle de Sartenejas, Caracas 1081-A, Venezuela*

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ABSTRACT

A 50-kDa-polypeptide band peripherally bound to retinal rod outer segment (ROS) membranes was purified by anion-exchange chromatography. When the 50-kDa protein was compared with purified arrestin-1, it was observed that: (1) both proteins comigrated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and were recognized by either anti-50-kDa protein polyclonal antibodies or anti-arrestin-1 monoclonal antibodies; (2) protein fragments and peptide fingerprint maps obtained following limited and complete proteolysis with specific proteases were very similar for both molecules; and (3) several chromatographically-purified tryptic peptides from the 50-kDa protein possessed the same amino acid composition as tryptic peptides deduced from the reported arrestin-1 primary structure. Consequently, arrestin-1 and the purified 50-kDa protein must correspond to variants of the same molecule. However, in contrast to arrestin-1 that associated to the ROS membranes only in the presence of light and ATP, the 50-kDa protein interacted with the ROS membranes in a light-independent manner, either in the presence or absence of ATP. These results clearly established that phosphorylated and illuminated rhodopsin is not the membrane anchor for this variant of arrestin-1.

Introduction

G protein-coupled receptors (GPCRs)[3] are a superfamily of proteins containing a core domain consisting of seven transmembrane α helices. GPCRs are activated in response to a large variety of extracellular signals including hormones, neurotransmitters, light, and odorants [1,2]. Receptor stimulation is followed by the activation of a heterotrimeric G protein and the subsequent activation of specific effector enzymes and ion channels, which ultimately yields changes in cellular physiology. To terminate signaling through the cascade efficiently, all activated intermediates, including the receptor, need to be inactivated. The inactivation of GPCRs, often termed desensitization, occurs in a two-step process. The first step involves a rapid phosphorylation of the activated receptor by a specific GPCR kinase, which results in only minimal desensitization; however, in the second step, phosphorylation does increase the affinity of the receptors for another group of proteins, known as arrestins. The binding of arrestin to phosphorylated and activated GPCRs quenches signal transduction via its apparent ability to decrease receptor/G protein coupling directly.

Arrestin was identified originally as an abundant protein in the bovine retina and was initially called S antigen [3]. This molecule is highly antigenic and has been detected in patients suffering from the inflammatory condition called uveitis [4]. Arrestin homologues have been identified in a large number of species and arrestins have also been found in nonretinal tissue. To date, four mammalian members of the arrestin family have been identified [5]. The prototypical arrestin, arrestin-1 (rod arrestin), regulates the light-dependent signal transduction of rhodopsin in rod photoreceptor cells. Arrestin-1 is also expressed at high level in cone photoreceptor cells [6]. Another retina-specific arrestin, arrestin-4 (cone arrestin or X-arrestin), is enriched in cones [7]. The nonvisual arrestins, arrestin-2 (β-arrestin-1) and arrestin-3 (β-arrestin-2), are expressed ubiquitously. These arrestins, β-arrestin-1 and 2, were so named because of their ability of desensitizing the β-adrenergic receptor, but they are involved in desensitization of most other members of the GPCR superfamily. However, β-arrestin-1 and 2 do demonstrate detectable preference for particular groups of receptors [8,9]. Several splice variants of the arrestin gene family have also

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* Corresponding author. Fax: +58 212 9063064.
E-mail address: jbubis@usb.ve (J. Bubis).

[1] Present address: Unidad de Estructura Molecular, Fundación Instituto de Estudios Avanzados – IDEA, Caracas, Venezuela.

[2] Present address: Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01655, USA.

[3] *Abbreviations used:* HPLC, high-performance liquid chromatography; NTB, 2-nitro-5-thiobenzoate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DEAE, diethylaminoethylcellulose, DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; PMSF, phenylmethylsulfonylfluoride; CM, carboxymethyl; TLCK, Nα-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, Nα-*p*-tosyl-L-phenylalanine chloromethyl ketone; ROS, rod outer segments; GPCRs, G protein-coupled receptors.

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been described [10,11], including variants of rod arrestin [12]. All of these variants are produced by alternative mRNA splicing at the 3′ end, producing truncated isoforms that have different biochemical characteristics than their full-length counterparts. A naturally occurring splice variant p44 of arrestin-1 has been cloned from bovine retina, in which the C-terminal 35 residues are replaced by a single alanine [13].

There has been a marked shift in the focus of research into arrestin function because it has become clear that they not only prevent signaling from GPCRs but also initiate and direct new signals from the very GPCRs that they desensitize. In general, arrestins are versatile regulators of cellular signaling expressed in every cell in the body, which directly interact with a great variety of diverse proteins, such as other classes of membrane receptors, Src tyrosine kinases, ubiquitin ligases, protein phosphatases, tubulin, Ca^{2+}/calmodulin, etc., and serve as scaffolds facilitating signaling in two mitogen-activated protein kinase cascades, leading to the activation of ERK1/2 and JNK3 [14,15]. It has also been demonstrated that arrestin plays an important role in the activation of an apoptotic-signaling pathway leading to retinal degeneration in *Drosophila* [16,17], as well as an antiapoptotic-signaling route in mouse embryonic fibroblasts [18].

Arrestin-1 is soluble in the dark but binds strongly to phosphorylated, photoexcited rhodopsin [19–21], and thereby competitively inhibits the binding of transducin [20]. As a result, the transducin-mediated activation of the rod photoreceptor cGMP phosphodiesterase (*aka* PDE6) is quenched [22]. Furthermore, light adaptation *in vivo* stimulates tyrosine phosphorylation in rod outer segments (ROS) of rat retina [23]. More specifically, illumination activates Src and promotes its association to ROS membranes by forming a complex with bleached rhodopsin and arrestin-1 [24]. Rod arrestin also binds to microtubules, and this interaction is increased in the dark [25] and plays an important role in arrestin-1 localization in photoreceptor cells [26]. In addition, a Ca^{2+}-dependent direct association between arrestin-1 and calmodulin has been described [27], which probably regulates the availability of arrestin-1 and calmodulin for other intracellular partners. Site-directed mutagenesis and spin labeling experiments have shown that microtubule and rhodopsin binding induce different conformational changes in arrestin-1 [28], indicating that arrestin-1 assumes diverse functional conformations in the cell. Sulfhydryl reactivity has also demonstrated that arrestin-1 is a flexible molecule that is able to adopt a variety of conformational states [29]. Thus, assorted subspecies of arrestin-1 [30,31] may reflect differences in their primary structure, may result from differentially regulated post-translational modifications, or may simply correspond to conformational variants of the same molecule with distinct functional properties. We report here the purification and characterization of a subspecies of rod arrestin, which is peripherally bound to ROS membranes in a light-independent manner.

Materials and methods

Materials

Reagents were purchased from the following sources: [γ-^{32}P] ATP (3000 Ci/mmol), New England Nuclear; Nα-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin, Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated α-chymotrypsin, carboxymethyl (CM)-Sepharose CL-6B, anti-rabbit IgG coupled to horseradish peroxidase, phenylmethylsulfonylfluoride (PMSF), leupeptin, sodium deoxycholate, Nonidet P-40, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), gel filtration molecular weight protein standards, monoclonal anti-α tubulin (clone DM 1A), monoclonal anti-β tubulin (clone TUB 2.1), sodium ortovanadate, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), O-phospho-L-serine, O-phospho-L-threonine, O-phospho-L-tyrosine, Sigma; diethylaminoethylcellulose (DEAE) DE 52, Whatman; superose 12, Polybuffer exchanger PBE 94, Polybuffer 96, Polybuffer 74, Sephadex G-25, heparin–agarose, DEAE–Sepharose, Pharmacia; anti-rabbit IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), Promega; biotinylated anti-mouse IgG horseradish peroxidase conjugate, Vectastain-ABC avidin immunodetection kit, Vector Laboratories; ECL luminol immunodetection system, Amersham; trifluoroacetic acid, phenyl isothiocyanate, Pierce; acetonitrile, EM Science; Bradford reagent, Bio-Rad; mouse monoclonal anti-phosphotyrosine (PY99) antibodies, rabbit polyclonal anti-phosphotyrosine (PY350) antibodies, Santa Cruz Biotechnology; rabbit polyclonal anti-phosphoserine antibodies, rabbit polyclonal anti-phosphothreonine antibodies, Invitrogen. Samples of purified bovine retinal arrestin-1, and mouse monoclonal antibodies S65-6 and S65-38 directed against the sequences containing residues 281–300 and 373–404 of arrestin-1, were generous gifts of Dr. Paul A. Hargrave, University of Florida, Gainesville, Florida, USA. Polyclonal antibodies against porcine heart type II regulatory subunit of the cAMP-dependent protein kinase were kindly provided by Dr. Susan S. Taylor, University of California, San Diego, USA. All other chemical compounds were analytical grade.

Preparation of ROS and washed membranes

Bovine eyes were obtained from the nearest slaughterhouse (Matadero Caracas, CA). Retinae were extracted in the dark, under red light, and were maintained frozen at −70 °C. ROS membranes were isolated in dim red light from frozen bovine retinas by flotation and subsequent centrifugation on discontinuous sucrose gradients [32]. Dark-depleted-ROS membranes were prepared by washing ROS with 5 mM Tris–HCl (pH 7.4), 2 mM EDTA, and 5 mM β-mercaptoethanol until no significant amount of peripheral proteins were released with the wash buffer [33]. ROS membranes and dark-depleted-ROS membranes were stored in the dark at −70 °C.

Purification of the 50-kDa polypeptide band

Illuminated ROS were isolated from frozen bovine retinae under room light, at 4 °C. All sucrose solutions were prepared in isotonic buffer [100 mM potassium phosphate (pH 6.8), 1 mM magnesium acetate, 5 mM β-mercaptoethanol, 0.1 mM PMSF]. Briefly, 200 retinae were thawed and extracted in 30% sucrose. The homogenate was centrifuged at 2987*g* for 6 min, at 4 °C, and the supernatant containing the crude ROS was decanted into a flask. This procedure was repeated three times. The ROS-containing solution was then diluted to 15% sucrose by slow addition of isotonic buffer, and the crude ROS sample was sedimented at 11,950*g* for 10 min, at 4 °C. The supernatant that resulted from this step ($S_{15\%}$) was stored at −20 °C for the preparation of soluble arrestin-1, and the pellet containing the ROS was suspended in 15% sucrose, divided into centrifuge tubes, underlayed with 0.64 M sucrose, and centrifuged for 10 min at 11,950*g* and 4 °C, to remove residual soluble proteins. The resulting supernatants ($S_{0.64M}$) were saved frozen at −20 °C for subsequent analyses, and the ROS-containing pellet was suspended in 0.64 M sucrose. Twelve three-step sucrose gradients containing 0.84, 1.00 and 1.20 M sucrose were prepared in Beckman ultraclear centrifuge tubes (25 × 89 mm), and the partially purified ROS sample was overlayed on the top of each gradient. The tubes were centrifuged at 140,000*g*, for 30 min, at 4 °C, without brake, in a Beckman L3-50 ultracentrifuge, using a SW 28 rotor, and purified ROS banded at the 0.84 M sucrose/1.00 M sucrose interface.

The purified illuminated ROS membranes were extracted with isotonic buffer by manually resuspending the membranes in a