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Immunological detection of arrestin, a phototransduction regulatory protein, in the cytosol of nucleated erythrocytes

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Cytosolic extracts of trout and turkey erythrocytes were tested for their immunoreactivity with polyclonal and monoclonal antibodies to retinal arrestin (S-antigen), a cytosolic protein of photoreceptor cells involved in the desensitization of rhodopsin After adsorption or immunoaffinity chromatography of the extracts, these antibodies specifically recognized a protein having a molecular weight similar to that of retinal arrestin Because the G-protein-mediated transduction systems, such as visual and β-adrenergic systems, display a high degree of structural and functional homology, the presence of arrestin-like proteins in non-photosensitive cells suggests that these proteins are involved in the transduction of chemical signals, with a possible role in receptor desensitization

Arrestin, Retinal S-antigen, Erythrocyte, β-Adrenergic transduction, Phototransduction, (Trout, Turkey)

1. INTRODUCTION

The transduction of many external stimuli as different as hormones, drugs or light into intracellular signals involves three identifiable components: a membrane receptor, which specifically recognizes and binds the stimulus, a GTP binding regulatory protein (G-protein) and an effector enzyme. These G-protein-mediated transduction systems display a high degree of structural, functional and regulatory homology [1]. For example, the light receptor R and βAR share extensive amino acid sequence and three-dimensional homology; the kinship of G-proteins from the two systems is revealed by their possible interchangeability; desensitization in the visual and hormonal systems involves receptor phosphorylation by specific cytosolic kinases (R kinase and βAR kinase, respectively), but βAR kinase is also capable of phosphorylating R in a light-dependent fashion, and alternatively R kinase can phosphorylate βAR.

Desensitization of rhodopsin, however, in addition to be initiated by kinase-mediated phosphorylation, needs for completion the subsequent binding of a cytosolic 48 kDa protein, variously termed 'S-antigen', '48K protein' or 'arrestin'. The 48K protein-rhodopsin complex blocks the activation of the effector enzyme by the G-protein, transducin, thereby arresting phototransduction [2] (thus the name arrestin) [3]. This 48K protein has been identified [4] as a previously described protein, abundant in the cytosol of photoreceptor cells, and having autoantigenic properties (thus the name S-antigen) [5]. Because this abundant protein induces autoimmune ocular inflammatory disease in laboratory animals [5–8], much information has been accumulated during the last 10 years concerning its structure. Moreover, due to its strong antigenic properties, several laboratories have successfully prepared monoclonal antibodies directed against well-defined antigenic sites of this protein. In part because of its regulatory role in phototransduction, but also with its immunocytochemical detection restricted to vertebrate and invertebrate photoreceptors [9,10], arrestin is considered to be a specific marker of photosensitive cells and of photoreceptor-derived cells of the pineal gland [5,11]. However, taking into account the strong structural and functional homologies shared by visual and hormonal transduction systems which involve G-proteins, the possibility arises that arrestin-like proteins could be present in non-photosensitive cells and could control the β-adrenergic transduction system. This hypothesis is supported by two sets of experimental data. Firstly, it has been recently shown that in a reconstituted system, retinal arrestin potentiates the desensitization of β-adrenergic receptor purified from hamster lung [12]. Secondly, the presence of arrestin-

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Abbreviations. βAR, β-adrenergic receptor; BSA, bovine serum albumin; PDE, cGMP phosphodiesterase, PMSF, phenylmethylsulfonylfluoride; R, rhodopsin, SDS-PAGE, SDS-polyacrylamide gel electrophoresis, TPCK, L-1-chloro-3-tosylamido-4-phenyl-2-butanone

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like protein in non-photosensitive cells is suggested by the detection of specific immunological reactivity in histological preparations of quail choroid plexus [13] and human cellular medulloblastoma cells [14].

Moreover, using immunofluorescence with monoclonal antibodies to arrestin on sections of fish, amphibian and avian organs, we observed in some specimens a positive immunoreactivity of the nucleated erythrocytes retained in the blood capillaries, suggesting that the S-antigen could also be present in nucleated erythrocytes. A recent observation that stimulation of fish erythrocytes by β-adrenergic catecholamines or by phorbol ester induces the phosphorylation of a cytosolic 48 kDa protein [15] appears as an additional argument since it has been shown that retinal arrestin can be phosphorylated by the retinal protein kinase C [16].

The data presented in this study demonstrate, in cytosolic extracts of trout and turkey erythrocytes, the presence of a protein having a molecular weight similar to that of retinal arrestin and which specifically reacted with polyclonal and monoclonal antibodies directed against several epitopes of retinal arrestin.

2. MATERIALS AND METHODS

2.1 *Preparation of erythrocytes*

The solutions used were (in mM)· Solution A: NaCl 145, KCl 4, $CaCl_2$ 5, $MgSO_4$ 1, Hepes 15, pH 7 5 Lysis solution B. KCl 4, $MgSO_4$ 1, $CaCl_2$ 5, Hepes 15, EDTA 1, PMSF 1, TPCK 0 1, pH 7 5. Solution C NaCl 10, KCl 4, $CaCl_2$ 5, $MgSO_4$ 1, Hepes 15, PMSF 1, TPCK 0.1, pH 7 5 Freshly drawn trout or turkey blood was washed 4 times in solution A and the buffy coat of leukocytes was carefully removed from the red blood cell pellet after each sedimentation The erythrocytes were suspended at 15% (v/v) in solution A + 5 mM glucose

2.2 *Processing of lysis and fractions*

After overnight storage at 4°C, the suspension was centrifuged and the erythrocytes were hypotonically lysed at 0°C by diluting 1 ml of compact cells into 10 ml of solution B. Insoluble material was removed by centrifugation (45000 × *g* for 15 min) and the cytosolic proteins contained in the supernatant were precipitated by 40% saturated $(NH_4)_2SO_4$ (0°C, sedimentation for 3 h followed by centrifugation at 45000 × *g* for 10 min) The pellet was resuspended in a minimal volume of solution C. A second precipitation was similarly performed The ammonium sulfate precipitates were then either chromatographically adsorbed on hydroxyapatite or onto an immunoaffinity support matrix A 20 ml column of hydroxyapatite (Serva, Heidelberg) was loaded with the extract (10 mg of protein). Three fractions were eluted with 50, 100 and 200 mM phosphate buffer (pH 7 4). The immunoglobulin fraction from a rabbit hyperimmune serum (L6) against bovine arrestin (purified as described in [17]) was coupled to cyanogen bromide-activated agarose beads (CNBr-activated Sepharose, Pharmacia) according to the recommendations of the manufacturer (7 mg of immunoglobulins per ml of beads). The solution (30 mg of protein) was passed through a 5 ml column of antibody beads with a flow rate of 10 ml/h After washing with 10 mM phosphate buffer, the proteins retained by the beads were eluted with a 200 mM glycine-HCl buffer, pH 2.5 at 4°C and the eluate was immediately neutralized by addition of 2 M Tris, 1 M KH_2PO_4 solution in the tubes Effluents were collected as 1 ml fractions

2 3 *Immunoassays*

The monoclonal antibodies used in this study have been produced by hybridomas from fusion of spleen cells from a BALB/c mouse immunized with purified bovine arrestin and NS-1 myeloma cells [18] Mapping of antibody epitopes on the amino acid sequence of the protein have allowed location of the sites recognized by the monoclonal antibodies S2D2, S6H8, S7D6, S8D8, S8D1 in the N-terminal region and the epitopes corresponding to the antibodies S9E2, S1A3, S10H9 in the C-terminus (Mirshahi, M. et al and Stiemer, R et al., in preparation) Antisera were raised in rabbits and rats immunized with native bovine arrestin, and in rats immunized with the synthetic peptide M (sequence of amino acids 303–320 of the protein) in Freund's adjuvant.

ELISA 96-well microplates were coated with protein solutions ($(NH_4)_2SO_4$ precipitates or undiluted chromatographic fractions) Plates were incubated with the rat or rabbit polyclonal or mouse monoclonal antibodies (diluted in PBS containing 1% BSA and 0.05% Tween 20) then with biotinylated goat antibody against immunoglobulins of the corresponding species, followed by streptavidin-biotinylated horseradish peroxidase complex (Amersham, England). *o*-Phenylenediamine-H_2O_2 was used as peroxidase substrate.

For SDS-PAGE, the immunoreactive fractions were pooled and concentrated by a two-step filtration procedure using first Ultrafree 30K filter (Millipore, Bedford, USA), and then microconcentrator Centricon filter (Amicon, Danvers, USA). Proteins were separated on 7.5% acrylamide gels and electrotransferred to PVDF membranes (Immobilon, Millipore, Bedford) Non-specific sites were blocked with 7 5% fat-free dry milk in PBS for 4 h. Immunoreactions were performed in a Miniblotter 25 (Immunetics, Cambridge, USA) using the same primary antibodies and biotine-streptavidin reagents as for ELISA 4-Chloro-1-naphthol was used to visualize the complexes.

3. RESULTS

The various polyclonal and monoclonal antibodies to arrestin slightly reacted in ELISA with $(NH_4)_2SO_4$ precipitated cytosolic extracts of trout and turkey erythrocytes.

To increase the concentration of the immunoreactive protein(s) ammonium sulfate precipitates were chroma-

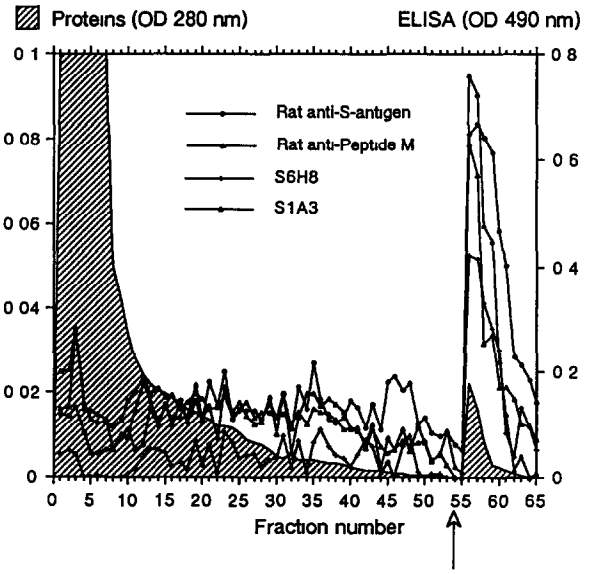

Fig 1 Immunoaffinity fractionation of soluble cytosolic proteins from trout erythrocytes on a column of rabbit antibody (L6) to bovine retinal arrestin coupled to CNBr-activated Sepharose. The fractions eluted with glycine-HCl buffer (arrow) positively react in ELISA with the antibodies to arrestin

tographically adsorbed onto hydroxyapatite or onto an immunoaffinity support matrix using a rabbit antibody to bovine retinal arrestin. Fig.1 shows the elution profile of trout erythrocyte cytosolic extract from the immunoaffinity column as tested by ELISA with various antibodies to arrestin. The effluents collected during the washing of the column with the 10 mM phosphate-buffered solution (fractions 1–54) contained a large amount of proteins which did not significantly react with antibodies. Conversely, the proteins retained by the beads and eluted with the acidic glycine buffer (start of elution indicated by the arrow) positively reacted with all the tested antibodies directed at epitopes in the N-terminal region of the protein, or at epitopes in the C-terminal region and with antisera against bovine arrestin or directed at the synthetic peptide M.

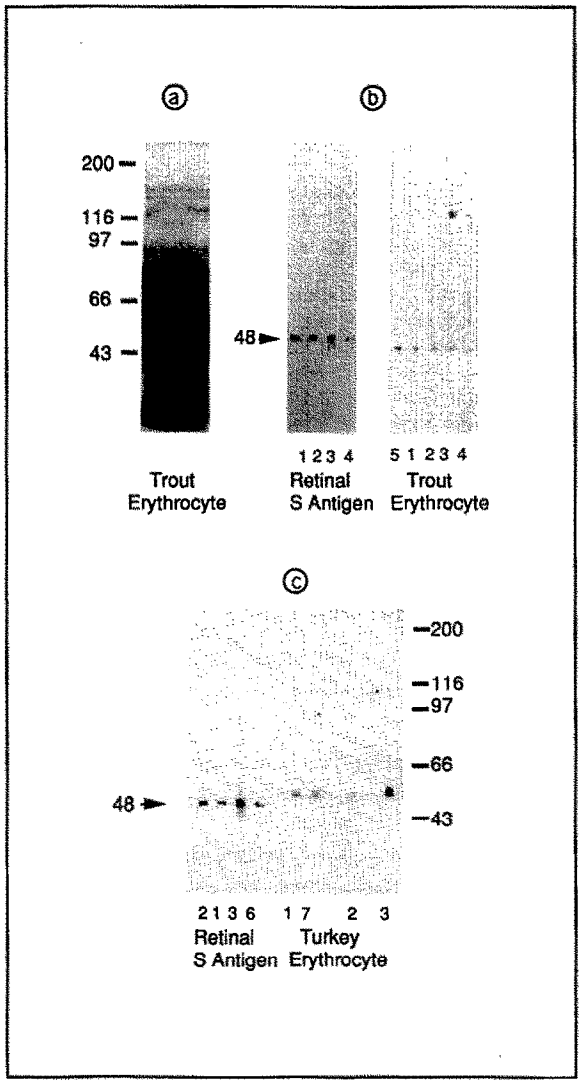

Fig.2. Immunological characterization of an arrestin-like protein in trout and turkey erythrocytes. (a,b) Trout erythrocyte cytosolic extract: (a) gel electrophoresis of total proteins (Coomassie blue stained) in the hydroxyapatite fraction; (b) immunoblots of the same hydroxyapatite fraction using different monoclonal anti-arrestin antibodies (to compare with immunoblots of purified arrestin from bovine retina). (c) Immunoblots of a hydroxyapatite fraction obtained from a turkey erythrocyte cytosolic extract. Monoclonal antibodies are indicated by lane numbers: antibodies directed at epitopes in the N-terminal region of arrestin are 1, 2, 5, 7 (S7D6, S2D2, S6H8, S8D1, respectively); those directed at epitopes in the C-terminal region are 3, 4, 6 (S9E2, S1A3, S10H9, respectively. Molecular mass markers are shown on the left (in thousands).

To characterize the reactive protein(s), eluates from hydroxyapatite and immunoaffinity columns were tested after migration of the proteins in SDS-PAGE. Fig.2a shows the total proteins contained in the hydroxyapatite fraction obtained from a trout erythrocyte cytosolic extract. Fig.2b illustrates, on the same fraction after transfer from the SDS gel to PVDF membrane, the immunological characterization of a protein by different monoclonal antibodies to arrestin. Similarly, fig.2c illustrates that in preparations from turkey erythrocytes, there was also a protein immunoreactive with antibodies to arrestin. Clearly all the monoclonal antibodies specifically recognize a polypeptide with a molecular weight similar to that of arrestin. The apparent molecular mass of the immunoreactive protein was slightly lower than 48 kDa in trout erythrocytes and slightly higher in turkey erythrocytes. It is noteworthy that an immunoreactive protein was not detected in similar extracts of human erythrocytes.

4. DISCUSSION

Immunocytochemical methods have allowed the characterization of arrestin as an abundant component of the cells involved in the reception of light: it has been localized in photoreceptor cells of vertebrates and invertebrates [9,10] and in photoreceptor-derived cells of the pineal gland [5,11]. Arrestin has been purified from the retina of several mammals [8]. Moreover cDNAs coding for this protein have been prepared both from bovine [19,20], human [21] and murine [22] retina and from the rat pineal gland [23].

In retinal photoreceptors, arrestin plays a regulatory role during phototransduction: it binds to photoexcited and phosphorylated rhodopsin and participates in the quenching of PDE activation. Two possible modes of action of arrestin have been proposed. One is that binding of arrestin to phosphorylated rhodopsin inactivates the receptor through competition with the α-subunit of transducin, impeding binding of this subunit to rhodopsin, thereby quenching its capacity to activate PDE [2]. It is noteworthy that amino acid sequence homologies have been found between arrestin and the α-subunit of transducin [19,23]. The second is that photoactivated rhodopsin catalyzes the formation of an activated form of arrestin which dissociates from rhodopsin and then binds to PDE, thereby inhibiting its activation [3,24].

Positive arrestin immunoreactivity has, however,

also been observed on histological sections in cells that are not known to be photosensitive: epithelium of the choroid plexus of the quail [13] and human medulloblastoma cells [14].

Our experiments show that turkey and trout nucleated erythrocytes contain a cytosolic protein which is similar to retinal arrestin in respect to their electrophoretic mobility in SDS-PAGE and their immunoreactivity with a panel of polyclonal and monoclonal antibodies. This panel includes antibodies directed at 3 different regions of retinal arrestin: highly conserved epitopes in the N-terminus, epitopes in the C-terminus, and the synthetic peptide M that reproduces a sequence of arrestin involved in the production of autoimmune uveoretinitis and pinealitis [25]. The fact that this sequence is not frequently encountered in other proteins [26] stresses the significant homology between retinal arrestin and the arrestin-like protein found in erythrocytes.

In recent experiments (to be published), we used the same procedures of protein fractionation and immunochemical analysis for extracts of several bovine organs, i.e. myocardium, liver, kidney, lung parenchyma and cerebellum, compared to the bovine retina and pineal gland. The fractions separated from all of these tissues reacted in ELISA with antibodies to arrestin, and contained a protein immunoreactive with the antibodies on Western blots. This protein displayed the same migration pattern in all bovine tissues including the retina, corresponding to a molecular mass of 48 kDa.

One can therefore suggest that the protein arrestin, or the members of a family of structurally related arrestin-like proteins, are indeed widely distributed in the body, and not restricted to photosensitive organs. The fact that arrestin has been initially detected only in photoreceptors and in pineal cells is probably due to its particularly high concentration in these cells. This peculiarity can be related to the great abundance of the individual components of the light transduction cascade, which represent more than 90% of the total protein content of the retinal rod outer segment.

As stressed above, the phototransduction cascade has striking similarities to other G-protein-mediated transduction systems such as the adenyl cyclase system. Thus it is possible that the adenyl cyclase system also has a protein analogous to arrestin, playing a similar role in negative regulation. Such a functional analogy is supported by a recent finding showing that in liposomes, retinal arrestin could bind to the phosphorylated β-adrenergic receptor and potentiate the desensitizing effect of βAR phosphorylation [12]. This fits in with the presence of arrestin or arrestin-like proteins in cells controlled by an active β-adrenergic adenyl cyclase transduction system (nucleated erythrocytes and cells in the tested bovine organs), and its absence in cells without such a control as human erythrocytes.

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