Arrestin from nucleated red blood cells binds to bovine rhodopsin in a light-dependent manner

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Using a panel of monoclonal antibodies, it has previously been demonstrated that the cytosol of nucleated red cells (trout and turkey) contains a protein similar to arrestin, a soluble protein found so far only in the photosensitive cells and which, by binding to photoexcited rhodopsin, inhibits the phototransduction process. The role of this arrestin-like protein in non-photosensitive cells is questionable. In this report we present evidence that partially purified red blood cell arrestin (RBC arrestin) behaves functionally like bovine retinal arrestin: it binds to phosphorylated bovine rhodopsin only when this receptor has been photoactivated. Thus RBC arrestin and bovine retinal arrestin are closely related both structurally and functionally. By analogy with the function of retinal arrestin, it is proposed that RBC arrestin is involved in desensitization of membrane transport proteins and/or adrenergic receptors.

Arrestin; Erythrocyte; β Adrenergic transduction; Na/H antiport; Phototransduction; Trout; Turkey

1. INTRODUCTION

The response of a retinal rod cell to a flash of light is mediated by a receptor/GTP-binding protein (rhodopsin/transducin) signal transduction system. Absorption of light by rhodopsin leads to conformational changes in the rhodopsin molecule [1]. This photoexcited rhodopsin activates the cGMP cascade of visual transduction and is then partially deactivated through its phosphorylation by a specific protein kinase (rhodopsin kinase)[2]. Binding of arrestin, a 48 kDa cytosolic protein, to the photoexcited and phosphorylated rhodopsin potentiates this inhibitory effect, leading to almost complete arrest of the transduction cascade. This process involves a competition between arrestin and transducin for interaction with rhodopsin. Arrestin does not bind to rhodopsin in the absence of phosphorylation of the photoexcited rhodopsin [3,4]. Partly because of its regulatory role in phototransduction and partly because its immunocytochemical detection is restricted to vertebrate and invertebrate photoreceptors, arrestin was considered to be a specific marker of photosensitive cells and photoreceptor-derived cells of the pineal gland [5,6]. Recently however, we have demonstrated[7] that turkey and trout nucleated red cells, and extracts of several bovine organs, contain a cytosolic protein which is similar to retinal arrestin as regards its electrophoretic mobility in SDS-PAGE and immunoreactivity with a panel of monoclonal and polyclonal anti-arrestin antibodies. This report demonstrates that the arrestin-like protein extracted from trout red blood cells (RBC) functionally behaves like retinal arrestin, i.e. the RBC arrestin binds to phosphorylated bovine rhodopsin only when it has been photoactivated.

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2. MATERIALS AND METHODS

2.1. *Preparation of RBC arrestin*

The first step in preparing arrestin-like protein from erythrocytes is to remove hemoglobin by ammonium salt precipitation. The protocole used to prepare and to lyse erythrocytes has previously been described [7].

2.2. *Processing of fractions*

Fractionation of soluble cytosolic proteins on a FPLC-gel filtration column. The FPLC column was loaded with the ammonium sulfate precipitate (1.5 mg protein/ml); flow rate 0.4 ml/min with elution buffer (20 mM Tris-HCl, 50 mM NaCl, pH 7.5). Total protein content and reactivity in ELISA were measured on all the fractions eluted as described in [7].

Immunoaffinity chromatography. The fractionation of a cytosolic extract from red cells, previously enriched by FPLC gel filtration, was obtained on a column of the monoclonal antibody S_9E_2 to bovine arrestin coupled to CNBr-activated sepharose. Methods as in [7].

2.3. *Immunoassays*

Antibodies: bovine arrestin is a single chain containing 404 amino-acids. Four monoclonal antibodies to arrestin were used in this study. Mapping of epitopes recognized by these antibodies on the amino-acid sequence of bovine retinal arrestin has allowed location of the epitopes for S_2D_2 and S_6H_8 in the N-terminal region, the epitope for

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S_9E_2 in the C-terminus and the epitope for M_4H_{10} in the sequence of amino-acid 283–302. ELISA and Western blot techniques using monoclonal antibodies have been previously described [7]. An immunometric assay has been developed allowing the protein sandwiched between a C- and a N-terminal monoclonal anti-arrestin antibody to be titrated very specifically : 96-well microplates were coated with the N-terminal anti-arrestin antibody S_6H_8; plates were incubated with cytosolic extracts, washed, then reincubated with the C-terminal monoclonal antibody S_9E_2 coupled with acetylcholinesterase [14] and finally washed. Ellman reagent was used to measure the acetylcholinesterase activity fixed on arrestin [15]. A standard curve was established by using different concentrations of bovine retinal arrestin.

2.4. *Experimental system used to assay the binding of RBC arrestin to bovine rhodopsin contained in disc membrane*

Rod outer segment (ROS) disc membranes were purified from fresh bovine retina [8] and stored under nitrogen at −80°C. Rhodopsin is by far the predominant protein in the disc membrane. Two types of material were obtained from the ROS. One batch of ROS was extensively washed in dark condition to remove retinal arrestin (washing solution : 5 mM Tris, 1 mM Mg, pH 7.5). The disc membranes containing non-activated, arrestin-free rhodopsin were then conserved (a). A second batch of ROS was illuminated, inducing the fixation of soluble retinal arrestin on photoactivated and phosphorylated rhodopsin. The rhodopsin-containing pellet was discarded. The supernatant, free of retinal arrestin (as shown in Fig. 3a lane 2) but containing other soluble compounds including rhodopsin kinase was kept (b). The binding of RBC arrestin to bovine rhodopsin was essayed first by mixing in the dark: non-activated arrestin-free rhodopsin (a), arrestin-free soluble compounds (b) and the RBC cytosolic extract containing RBC arrestin (all freshly prepared), 3 mM ATP and 0.1 mM GTPγS (to keep transducin in the active form, not competing with arrestin). In a second step the above mixture was either maintained in the dark or exposed to light.

2.5. *Binding of RBC arrestin to solubilized rhodopsin adsorbed to ELISA Plates*

ROS disc membranes were extensively washed in the dark (to wash out internal arrestin). They were illuminated in the presence of an arrestin-free supernatant but containing rhodopsin kinase and supplemented with ATP and GTPγS. Solubilization of the photoactivated and phosphorylated rhodopsin, theoretically free of bovine retinal arrestin, was performed in a solution containing 5 mM Tris-HCl, 1 mM $MgCl_2$, 0.5% Triton X-100 'reduced' Sigma. Solubilized rhodopsin was adsorbed overnight to ELISA plates (4°C) in ELISA coupling buffer pH 9.6. The fixed rhodopsin was then incubated with RBC extract freshly obtained by FPLC-gel filtration, or with a solution containing bovine retinal arrestin. Reactivity in ELISA with monoclonal antibodies was then tested.

3. RESULTS AND DISCUSSION

Figure 1a shows the elution profile of trout erythrocyte cytosolic extract from FPLC gel filtration. The effluents collected in fractions 1–15 contained a large amount of proteins which did not significantly react in ELISA with either C- or N-terminal directed monoclonal antibodies. The main immunological response occurs in the region where proteins smaller than 60 kDa are eluted (fractions 18–21). Elution and immunological reactivity profiles of turkey erythrocytes were similar to those of trout erythrocyte (not shown). The proteins contained in these fractions enriched by FPLC were characterized after migration in SDS-PAGE. Figure 1b shows the total proteins, stained by Coomassie blue, contained in turkey (lane 1) and trout (lane 2) erythrocytes : in these fractions the high molecular mass proteins have been discarded but there are still many proteins in the 20–60 kDa range. However, on the same fractions after transfer from the SDS gel to PVDF-immunoblot membrane, only one protein was immunoreactive with antibodies to arrestin (Fig. 1c). The apparent molecular mass of the immunoreactive protein was slightly lower than 48 kDa in trout erythrocytes, and higher in turkey erythrocytes. An additional characterization of the arrestin-like protein of these fractions was obtained by developing an immunometric test allowing the protein sandwiched between a C- and an N-terminal monoclonal anti-arrestin antibody to be titrated very specifically as illustrated in Fig. 3b. Further purification of the arrestin-like protein was carried out by immunoaffinity chromatography using the monoclonal antibody S_9E_2 coupled to a cyanogen-bromide-activated gel. The elution profile is shown in Fig. 2a : the fractions corresponding to peaks 1 and 2, collected during the washing of the column, contained a large percentage of the total proteins which did not react with anti-arrestin antibodies (Fig. 2b). Conversely, the small amount retained by the column and eluted at low pH showed a very high arrestin-like immunoreactivity. Thus the arrestin-like protein was specifically retained by the antibody fixed on the gel, and the enrichment with the affinity column is very efficient. Unfortunately an irreversible aggregation of the arrestin protein was observed under elution at pH 2.5 as shown by Western blots in which only bands of high molecular mass were seen (not shown). Consequently, to test whether the arrestin-like protein found in RBC can bind to rhodopsin and whether this binding is light-induced, we used extracts only enriched by FPLC but containing the protein in the native form.

The experiments were performed with extracts from trout RBC and using the reconstituted experimental system described in section 2. Rod outer segment disc membranes from bovine retina, washed in the dark and thus containing rhodopsin in the non-activated form, were suspended in a medium containing both trout RBC arrestin and soluble compounds allowing rhodopsin phosphorylation (rhodopsin kinase and ATP). It is expected that absorption of light by rhodopsin in disc membranes will lead to conformational changes in the rhodopsin molecule making it available as a substrate for the kinase and then allowing the light-induced binding of arrestin. The data are similar whether obtained from immunoblot (Fig. 3a), by ELISA (Fig. 3b) or by an immunometric assay which allows very specific titration of the arrestin-like protein (Fig. 3c). They show that almost all of the RBC arrestin contained in the medium in contact with dark membrane disappears from the medium during the illumination period indicating that RBC arrestin, like retinal arrestin, binds to rhodopsin when this protein becomes photoactivated

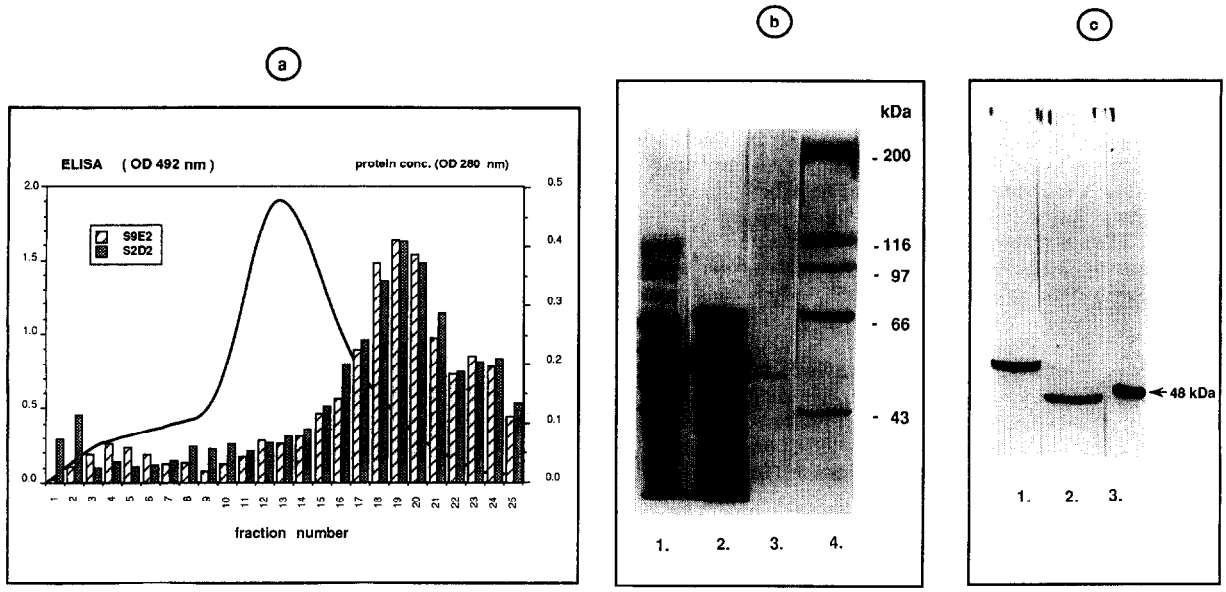

Fig. 1(a). Fractionation of soluble cytosolic proteins from trout erythrocytes on a FPLC-gel filtration column. The reactivity in ELISA of the different fractions was measured with two monoclonal anti-arrestin antibodies (S_2D_2 = N-terminal, S_9E_2 ⊥ C-terminal). (b) Gel electrophoresis of total proteins (Coomassie blue stained) contained in pooled arrestin-enriched fractions of RBC turkey (1) and trout (2) cytosolic extracts; lane 3: bovine purified arrestin; lane 4 : molecular mass markers. (c) Immunoblots of the same fractions (reactivity with the antibody S_6H_8). A similar result was obtained by using the other monoclonal antibodies.

and phosphorylated. Conversely the control disc membranes, not illuminated, do not fix RBC arrestin.

This result was confirmed in another type of experiment where solubilized rhodopsin, previously photoactivated and phosphorylated, was adsorbed on polyethylene microplates. It can be observed, as shown in Fig. 4, that trout RBC arrestin, like bovine retinal arrestin, binds to the rhodopsin-coated surface.

Our data suggest that RBC arrestin could have a functional role closely related to that of retinal arrestin. However, since red blood cells are not photosensitive cells, a target other than rhodopsin must be identified.

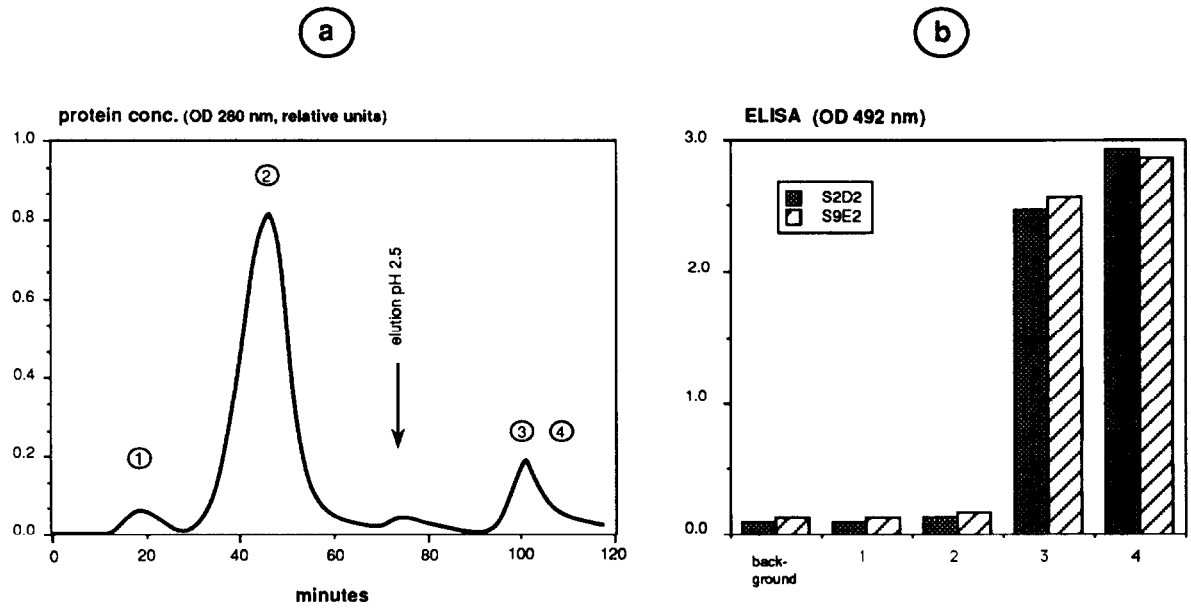

Fig. 2. Immunoaffinity chromatography. Fractionation of a cytosolic extract from RBC turkey, previously enriched by FPLC-gel filtration, on a column of the monoclonal antibody S_9E_2 to bovine arrestin coupled to CNBr-activated Sepharose. (a) Elution profile of proteins; (b) Immunoreactivity with C- and N-terminal monoclonal antibodies to arrestin. The fractions eluted with acidic buffer positively react in ELISA with both antibodies.

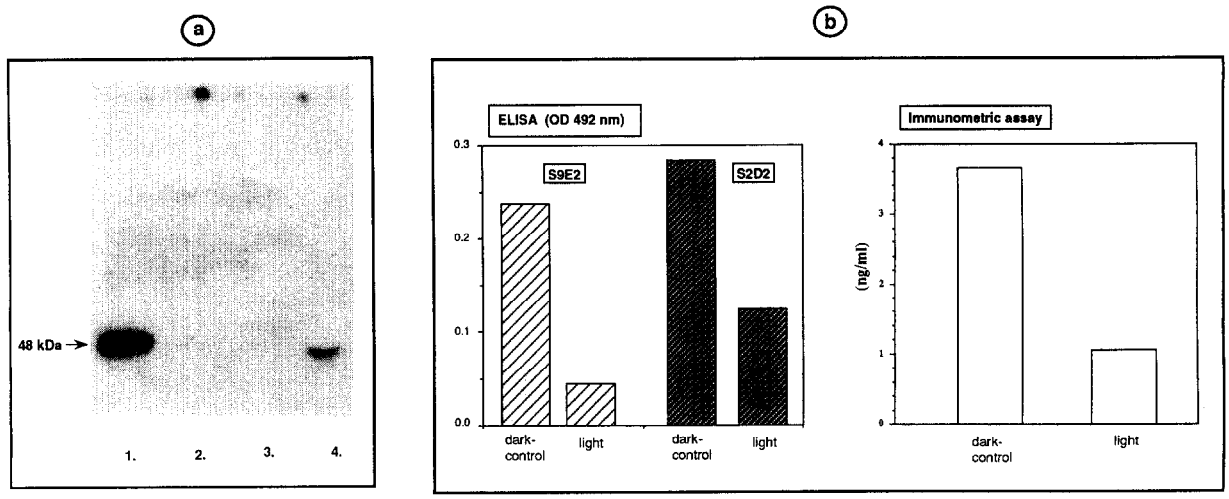

Fig. 3. Experiments showing the light-dependent binding of trout RBC arrestin to ROS disc membranes. (a) Immuno-detection of the RBC arrestin in the supernatants obtained from a binding assay experiment in which disc membranes were illuminated (lane 3) or kept in the dark (lane 4). After electrophoretic migration in SDS-PAGE, proteins were electro-transferred to PVDF membranes and incubated with an anti-arrestin antibody (in this experiment : the C-terminal monoclonal S_9E_2). The labeling of the antibody was obtained by fixation of radiolabeled protein A. This autoradiography shows the presence of RBC arrestin in the supernatant under dark control conditions (lane 4) and its disappearance after illumination (lane 3). Lane 2 shows that no residual endogenous retinal arrestin could be detected in the supernatant before RBC arrestin was added for the experiment. Lane [1]: bovine retinal arrestin as control. (b) Reactivity in ELISA of the supernatants, with or without illumination, measured either with two different monoclonal antibodies (S_9E_2 = C-terminal; S_2D_2 = N-terminal) as described previously [7] or by the immunometric assay.

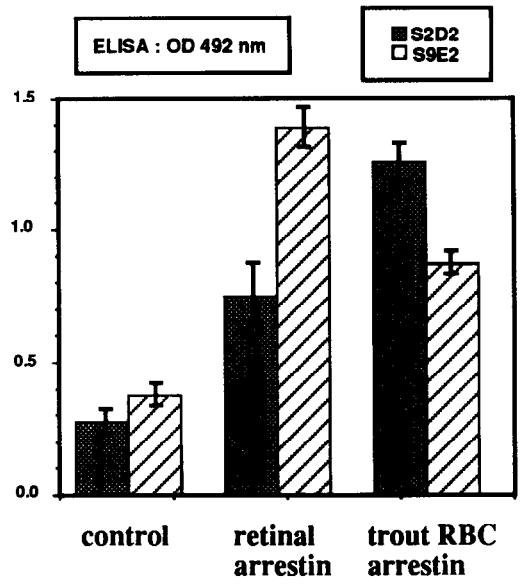

Fig. 4. Experiments showing the light-dependent binding of trout RBC arrestin to solubilized rhodopsin, previously photoexcited and phosphorylated, and then adsorbed to ELISA plates. It can be observed that the fixed rhodopsin incubated in the absence of arrestin (rhodopsin-control), reacts significantly in ELISA with the monoclonal antibodies S_2D_2 and S_9E_2: it is due to the tight binding of some endogenous arrestin to residual photoactivated rhodopsin due to incomplete dark adaptation at the time of slaughtering. Addition of either trout RBC arrestin or bovine retinal arrestin in the medium causes a large increase in the immunological signal, indicating the binding of both types of arrestin to the previously photoactivated and phosphorylated rhodopsin.

During the course of this work it has been reported that a complementary DNA from an arrestin-like protein was cloned in bovine brain [9], supporting our previous observation that arrestin-like proteins have a widespread distribution [7]. This arrestin-like protein has been expressed and shown to inhibit β-receptor function. Thus, the authors propose that it is involved in β-receptor desensitization in a similar way as retinal arrestin is involved in rhodopsin desensitization. Taking into account the analogies between visual and β adrenergic systems [10], this suggestion appears very likely. In trout RBC, however, no desensitization of β-receptors is observed over several hours whereas a β adrenergic-dependent membrane transport system (Na^+/H^+ antiporter) is very rapidly (2 min) and extensively desensitized [11]. Similarly in turkey red blood cells, the β adrenergic-dependent Na/K/Cl co-transport becomes desensitized [12]. Thus the possibility arises, as previously proposed [13], that arrestin-like proteins found in non-photosensitive cells are regulatory elements involved in the desensitization of various types of membrane proteins such as transport proteins and/or receptors .

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