Synthetic phosphopeptide from rhodopsin sequence induces retinal arrestin binding to photoactivated unphosphorylated rhodopsin

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Received 30 January 1995

Abstract A synthetic heptaphosphopeptide comprising the fully phosphorylated carboxyl terminal phosphorylation region of bovine rhodopsin, residues 330–348, was found to induce a conformational change in bovine arrestin. This caused an alteration of the pattern of limited proteolysis of arrestin similar to that induced by binding phosphorylated rhodopsin or heparin. Unlike heparin, the phosphopeptide also induced light-activated binding of arrestin to both unphosphorylated rhodopsin in disk membranes as well as to endoproteinase Asp-N-treated rhodopsin (des 330–348). These findings suggest that one function of phosphorylation of rhodopsin is to activate arrestin which can then bind to other regions of the surface of the photoactivated rhodopsin.

Key words: Arrestin; S-antigen; G-protein-linked receptor; Phosphorylation; Vision; Rhodopsin

1. Introduction

Arrestins are a class of proteins that participate in the deactivation of G-protein-coupled receptors. Once such a receptor has been activated it can activate a G-protein that can in turn influence the activity of a cell-specific target protein. The receptor is turned off – prevented from continuing to activate its G-protein – via phosphorylation by a protein kinase and subsequent binding of an arrestin. In a well-studied system of this kind, the visual receptor rhodopsin is photoactivated, phosphorylated by rhodopsin kinase, and bound by retinal arrestin (reviewed in [1]).

Retinal arrestin is a 45 kDa protein that has been best studied from cattle retinal rod cells (reviewed in [2]). Arrestin has been shown to occur widely phylogenetically throughout the vertebrates and invertebrates. The binding of arrestin to membrane-bound photoactivated phosphorylated rhodopsin has been detected directly by centrifugation [3], by a column binding assay [4], and spectrophotometrically [5]. Upon binding, arrestin stabilizes rhodopsin's photoproduct Metarhodopsin II. There is a high activation energy for this binding that indicates a large transient chemical change associated with the process [5]. The effect of arrestin binding can also be observed by its inhibition of light-stimulated cGMP-phosphodiesterase activity.

It has been recognized that phosphorylation of the activated receptor greatly enhances arrestin binding, and that binding is minimal to the unphosphorylated receptor [4,6–8]. In the present work, we show that in the presence of a synthetic phosphorylated peptide from rhodopsin's carboxyl-terminal sequence, arrestin can bind to photoactivated unphosphorylated rhodopsin. We demonstrate that binding of the phosphopeptide to arrestin causes a change in the conformation of arrestin, and that it is this new conformation of arrestin that is capable of binding to photoactivated rhodopsin. Other G-protein-linked receptors may similarly activate their corresponding arrestins which can then bind to other regions of the surface of the activated receptor.

2. Materials and methods

2.1. Purification of bovine arrestin

Arrestin was purified from bovine retinas by a modification of the method of Buczylko and Palczewski [9]. The extract containing arrestin was submitted to DEAE-cellulose chromatography at pH 7.0 rather than at pH 7.5. The column was washed with 1 liter of the buffered 15 mM NaCl. Arrestin was then eluted, using 1 liter of buffered 55 mM NaCl, directly onto the heparin-agarose column. Arrestin was eluted from the heparin-agarose column with a phytic acid gradient, dialyzed, again loaded on a heparin-agarose column and eluted with 400 mM NaCl in buffer as previously described [9]. These modifications shorten preparation time by 1 day and result in production of arrestin in high yield and purity. Approximately 25 mg of purified arrestin can be obtained from 100 bovine retinas.

2.2. Preparation of bovine rod outer segment membranes

All operations were carried out at 4°C under dim red light unless otherwise specified. Bovine retinas (Lawson, Inc., Lincoln, NE) were used to prepare rod cell outer segments (ROS) by sucrose gradient centrifugation [10]. Membranes were stored frozen prior to use.

2.3. Phosphorylation of rhodopsin

ROS were suspended in 100 mM potassium phosphate, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT (pH 7.0) to a final concentration of 13.2 μM rhodopsin. The ROS were brought to 30°C and ATP was added to 3 mM. The sample was illuminated for 1 h at 30°C with a 150 watt flood lamp from about 15 cm. The ROS were regenerated by adding a 3-fold molar excess of 11-*cis* retinal and incubating in the dark overnight [10].

2.4. Preparation of bovine ROS disk membranes

ROS membranes were prepared as above. Intact disk membranes were prepared from these ROS by Ficoll flotation [11].

2.5. Limited proteolysis of rhodopsin

Membrane-bound rhodopsin lacking its carboxyl-terminal sequence 330–348 was prepared by digestion of ROS disk membranes with

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Abbreviations: cGMP, guanosine 3′:5′-cyclic monophosphate; DEAE-cellulose, diethylaminoethyl cellulose; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; HEPES, (*N*-[hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); PDE, cGMP-phosphodiesterase; PMSF, phenylmethylsulfonyl fluoride; ROS, rod outer segment; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 7P[330–348], rhodopsin peptide of sequence 330–348 in which all seven serine and threonine residues are phosphorylated.

SSDI 0014-5793(95)00225-1

endoproteinase Asp-N [12]. Conversion of full-length rhodopsin to a lower molecular weight was confirmed by SDS-PAGE.

2.6. Limited proteolysis of arrestin

The limited proteolysis was carried out using the method of Palczewski et al. [13]. Arrestin at 0.5 mg/ml in 10 mM HEPES, 100 mM NaCl, 1 mM DTT, 0.1 mM $CaCl_2$ (pH 7.5) was digested with 3% TPCK-treated trypsin (Sigma) with no additions, with heparin (100 μg/ml), with phosphopeptide (1.0 mg/ml), or with unphosphorylated peptide 330–348 (1.0 mg/ml). Samples were withdrawn at 10 min after the addition of trypsin with the 0 time sample taken before adding trypsin. The digested aliquots were mixed with an equal volume of 20 mM benzamidine and 1 mM PMSF in the reaction buffer without DTT and with 0.5% isopropyl alcohol (used to dissolve the PMSF). The denaturing buffer was added and the samples were incubated at room temperature for 30 min. The samples were immediately submitted to SDS-PAGE.

2.7. Binding of arrestin to membrane-bound rhodopsin assessed by gel filtration

Twenty chromatography columns (Poly-Prep, Bio-Rad), supported on an acrylic rack, were filled with 2.0 ml of Sepharose 2B resin [14]. Columns were equilibrated with phosphate reaction buffer (100 mM sodium phosphate (pH 7.0), 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT). All steps of the reaction and analysis were performed at room temperature. ROS disk membranes for the binding studies were suspended in the reaction buffer at a rhodopsin concentration of 50 μM. Prior to use the ROS disk membranes were sonicated under dim red light under a stream of N_2 for 2 min (Microzoon ultrasonic cell disrupter, Heat Systems-Ultrasonics, Inc., NY, using a 2 mm sonicator tip, at 45% maximum output). Reaction mixtures (200 μl) were prepared containing a final rhodopsin concentration of 10 μM and measured amounts of arrestin and other effectors. Illuminated reaction mixtures were exposed to flood lights at 60 cm for 2 min. Dark reaction mixtures were held in the dark for the same time. All experimental samples were submitted to gel filtration at the same time. Immediately following illumination, each reaction mixture was applied to a column under dim red light and the eluant was discarded. Phosphate buffer (400 μl) was carefully applied, allowed to drain, and the eluant discarded. Subsequent 500 μl aliquots of buffer were applied and the eluants were collected and examined by SDS-PAGE. Membranes and bound arrestin were found in the first eluant collected while the unbound arrestin was eluted in later fractions.

2.8. SDS-PAGE

SDS-PAGE was performed under non-reducing conditions according to the method of Laemmli [15]. Aliquots of samples from the Sepharose columns were mixed 1:1 with 2× concentrated SDS-PAGE sample buffer and allowed to denature for 30 min at room temperature prior to applying 50 μl aliquots for SDS-PAGE. Acrylamide slab gels (12%, 1.5 mm thickness, containing 10 wells) were run in a minigel apparatus (Model SE 250, Hoeffer Scientific) for 1 h at 150 V. Gels were stained overnight with a filtered solution of Coomassie brilliant blue R-250 (Fisher) in 1:4.5:4.5 (v/v/v) acetic acid/methanol/water (4 h to overnight). Gels were destained using 1:3:6 (v/v/v) acetic acid/methanol/water.

2.9. Densitometry of SDS-PAGE gels

Densitometry was performed on destained SDS-PAGE gels on an electronically stabilized light table using a Microscan 1000 (Technology Resources, Inc.) fitted with a linear photodiode array to digitize the image. The digitized image was visualized and stored using TriScan software, and quantified using NIH Image Analysis software (version 153_nonfpu).

2.10. Peptide synthesis, purification and characterization

Peptides were synthesized from Boc-amino acids by standard solid-phase methods using an automated synthesizer (Applied Biosystems 431A). Peptides synthesized were residues 330–348 from bovine rhodopsin sequence which comprises the carboxyl terminal phosphorylation site – DDEASTTVSKTETSQVAPA. The fully phosphorylated phosphopeptide was synthesized by solid phase methods using t-boc phosphono esters of serine and threonine as previously described [16,17]. Preparative purification of peptides was achieved by reversed phase HPLC, using a 0–40% gradient of acetonitrile in 0.05% acetic acid on a Partisil 10 ODS-3 column (Whatman). Peptides were examined by analytical HPLC for purity and were determined to have the correct amino acid composition by acid hydrolysis with 6 N HCl followed by amino acid analysis. Molecular weight was verified by time-of-flight mass spectral analysis (Vestec).

3. Results and discussion

The most characteristic property of retinal arrestin is its light-dependent binding to phosphorylated rhodopsin [3,18]. Arrestin exhibits only negligible binding to phosphorylated rhodopsin in the dark, and its binding is greatly enhanced when rhodopsin is photoactivated (Fig. 1, lanes 1,2; [4,7,18]). Not only is photoactivation of rhodopsin necessary, phosphorylation is also required. Unphosphorylated rhodopsin in ROS disk does not bind arrestin, and binding is only slightly enhanced when rhodopsin is illuminated (Fig. 1, lanes 3,4). However, when the synthetic phosphopeptide that represents rhodopsin's carboxyl-terminal phosphorylation region (residues 330–348) is present, arrestin's binding to photoactivated rhodopsin is considerably enhanced (Fig. 1, lanes 5,6), reaching more than 50% of the level of binding to photoactivated phosphorylated rhodopsin (Fig. 1, lane 2). It is important that the rhodopsin synthetic peptide be phosphorylated. When unphosphorylated peptide 330–348 is incubated with arrestin and rhodopsin, it fails to stimulate light-dependent binding of arrestin (Fig. 1, lanes 7,8). These observations suggest that the free synthetic phosphopeptide of rhodopsin is able to substitute for the role of the phosphorylated carboxyl-terminal of membrane-bound rhodopsin in stimulating arrestin to bind to photoactivated rhodopsin. In fact, rhodopsin's carboxyl-terminal region can be eliminated without effect in these experiments as long as the phosphopeptide is present. Rhodopsin, whose carboxyl-terminal 330–348 has been removed by endoproteinase Asp-N digestion, binds arrestin in a light-dependent manner in the presence of the phosphopeptide (Fig. 1, lanes 9–12). Heparin and phytic acid, acidic compounds that also bind to arrestin [13], do not promote binding to photoactivated unphosphorylated rhodopsin (data not shown). The free phosphopeptide is not as effective as the rhodopsin-bound phosphopeptide in promoting arrestin binding (compare Fig. 1, lanes 2 and 6), presumably due to the peptide's ability to dissociate from the complex. (We have found that performing this binding assay on a column preequilibrated with phosphopeptide increases the amount of arrestin binding, but limited phosphopeptide availability precludes this method for routine analysis.)

The amount of phosphorylated peptide required to cause arrestin binding to photoactivated rhodopsin was determined using the column binding assay over a range of phosphopeptide concentrations (Fig. 2). The amount of arrestin bound to a standard amount of rhodopsin was measured by densitometry of the stained SDS-polyacrylamide gel. The K_{50} for the phosphopeptide was determined to be 36 μM at 6.4 μM arrestin.

It is reasonable to propose that the rhodopsin phosphopeptide achieves its effect by binding to arrestin and changing arrestin's conformation. We investigated this hypothesis by investigating the effect of the phosphopeptide on the limited proteolysis of arrestin. When arrestin binds to photoactivated phosphorylated rhodopsin or to heparin, the pattern of limited

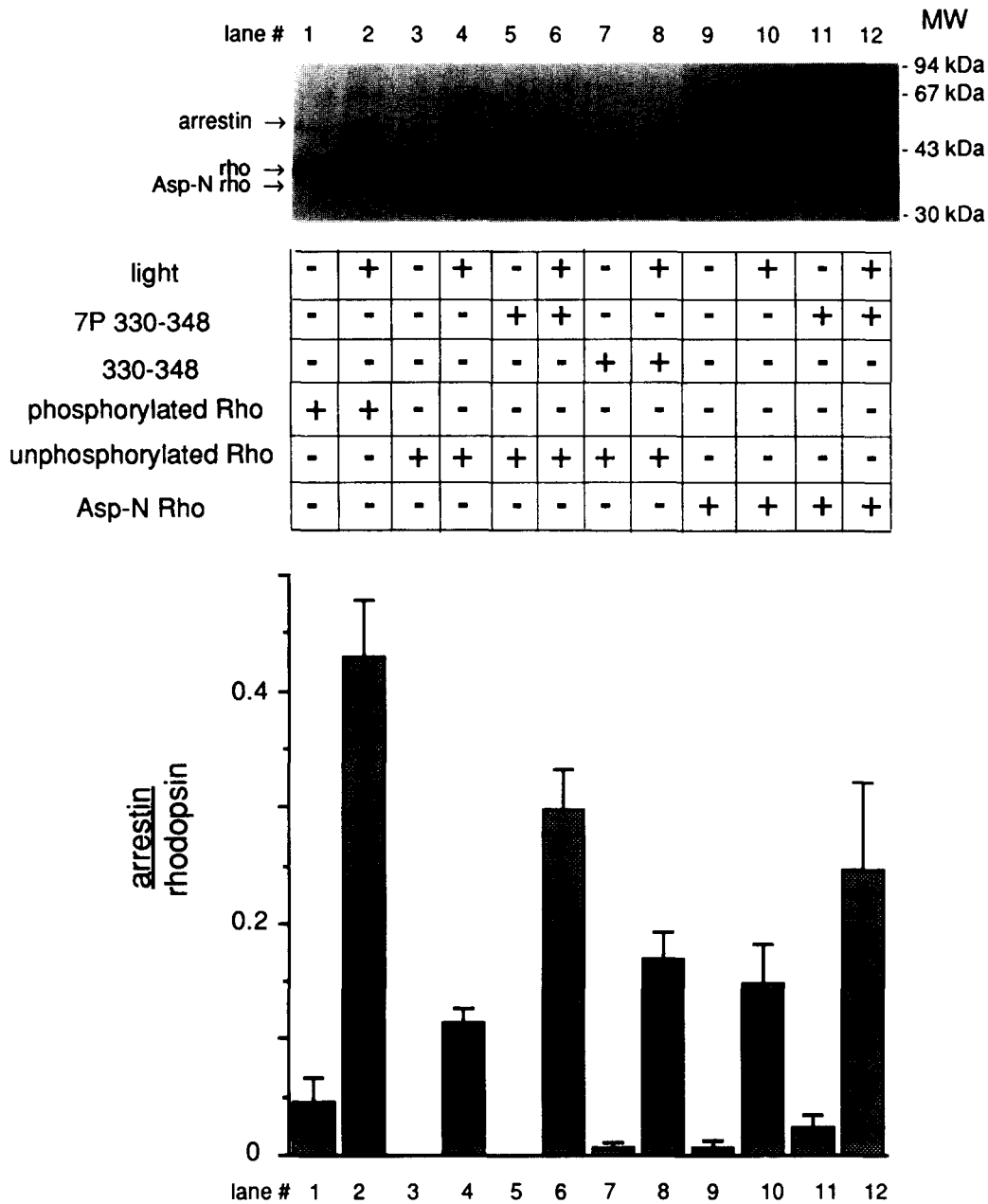

Fig. 1. Binding of arrestin to light-activated unphosphorylated rhodopsin in the presence of rhodopsin's heptaphosphopeptide. Sonicated ROS disk membranes containing rhodopsin were incubated in the presence of arrestin as described in section 2. The reaction mixture was submitted to Sepharose 2B column chromatography and the void volume containing the rhodopsin vesicles and protein bound to them was collected and submitted to SDS-PAGE. Coomassie-blue stained gels from a representative set of 12 experiments are shown in the top panel. The conditions for each experiment are listed in the table in the central panel below each lane: the presence (+) or absence (−) of rhodopsin in ROS disk membranes, phosphorylated rhodopsin in ROS, endoproteinase Asp-N-digested rhodopsin in ROS disk membranes, light, synthetic rhodopsin peptide [330–348], phosphorylated peptide 7P[330–348]. The bottom panel shows the amount of arrestin bound to rhodopsin as determined by gel densitometry (bars indicate standard error of the mean for between 4 and 10 samples).

proteolysis of arrestin by trypsin is altered [13]. We have examined the effect of the phosphopeptide on arrestin by limited proteolysis. Arrestin was first digested with trypsin either with no additions (Fig. 3, lane 2) or in the presence of unphosphorylated peptide 330–348 (Fig. 3, lane 3). Virtually identical patterns were obtained, consistent with the conclusion that the unphosphorylated peptide has no effect on arrestin's conformation. This is in contrast with the results obtained in the presence of heparin (Fig. 3, lane 4) or phosphopeptide (Fig. 3, lane 5). Here one observes an increase in the rate of proteolysis, as shown by the more rapid disappearance of full-length arrestin, and a decrease in the number of different digestion products. The pattern of limited proteolysis of arrestin in the presence of photoactivated phosphorylated rhodopsin in ROS was similar to that obtained with phosphopeptide and heparin (data not shown; [13]).

In conclusion, the data presented support the hypothesis that the phosphopeptide induces a conformational change in

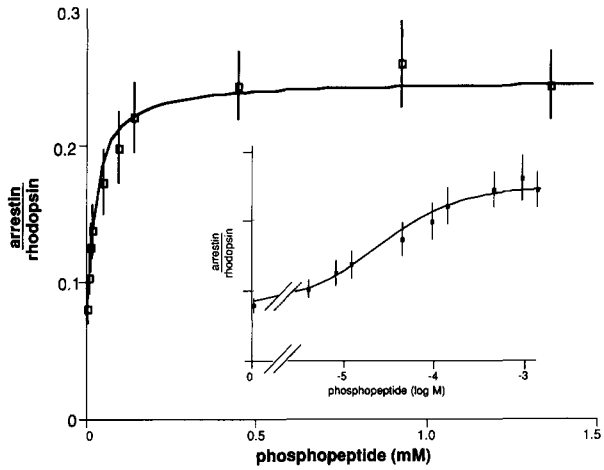

Fig. 2. Effect of amount of rhodopsin heptaphosphopeptide 330–348 on the binding of arrestin to photoactivated rhodopsin. The amount of arrestin bound per amount of rhodopsin in column binding experiments (as determined from gel densitometry) is plotted vs. the amount of 7P[330–348] in the reaction mixture. In these experiments the columns were not pre-equilibrated with phosphopeptide. *Inset:* The same data is plotted with log [phosphopeptide] on the X-axis.

arrestin. This conformational change is similar to that induced by the polyanion heparin so that our data are in reasonable accord with the model proposed by Palczewski and colleagues [13]. However, the current data indicate that only the phosphopeptide causes the conformational change that leads to enhanced binding of arrestin to photoactivated unphosphorylated rhodopsin. The phosphopeptide also induces binding to photoactivated endoproteinase Asp-N treated

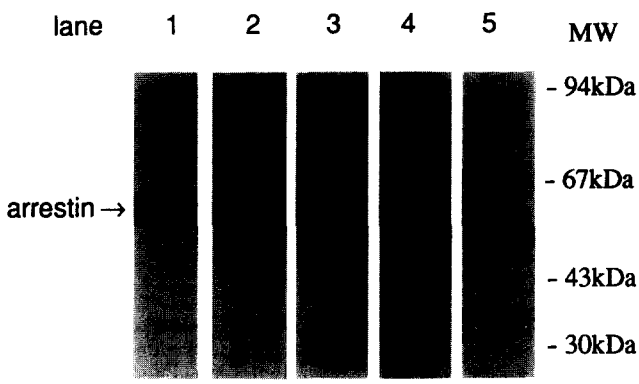

Fig. 3. Limited proteolysis of arrestin with TPCK-treated trypsin in the presence of unphosphorylated peptide 330–348, heptaphosphopeptide (330–348), or heparin. Samples from the limited proteolysis of arrestin were taken at the indicated times (see section 2) and submitted to SDS-PAGE. Undigested arrestin is shown in lane 1. Limited proteolysis for 10 min of: arrestin only (lane 2); arrestin plus unphosphorylated 330–348 peptide (lane 3); arrestin plus heparin (lane 4); arrestin plus 7P[330–348] (lane 5).

rhodopsin, a modified rhodopsin that is lacking the phosphorylation region (330–348). This demonstrates that the phosphorylated region of rhodopsin is not required per se for binding arrestin. The phosphorylated region of photoactivated rhodopsin appears to serve the purpose of promoting a conformational change in arrestin that enables arrestin to recognize and bind to other regions of photoactivated rhodopsin. Such regions appear to include rhodopsin's third cytoplasmic loop and possibly its first cytoplasmic loop [14]. By analogy, one may suggest that one function for the phosphorylation of other G-protein-linked receptors is to promote binding of their respective arrestins.

Acknowledgements: We would like to thank Drs. D. Wayne Bolen, K. Palczewski and K.P. Hofmann for their helpful discussions. Supported in part by research grants EY06225 and EY06226 from the National Eye Institute of the National Institutes of Health, a grant from the International Human Frontier Science Program Organization, and an unrestricted departmental award from Research to Prevent Blindness, Inc. P.A.H. is Francis N. Bullard Professor of Ophthalmology.

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