Functional Differences in the Interaction of Arrestin and Its Splice Variant, p^{44}, with Rhodopsin†

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ABSTRACT: Arrestin quenches signal transduction in rod photoreceptors by blocking the catalytic activity of photoactivated phosphorylated rhodopsin toward the G protein, transducin (G_t). Rod cells also express a splice variant of arrestin, termed p^{44}, in which the last 35 amino acids are replaced by a single Ala. In contrast to arrestin, this protein has been reported to bind to both the phosphorylated and nonphosphorylated forms of the activated receptor. In this study, we analyzed formation of the rhodopsin−p^{44} complex *in vitro*. Like arrestin, p^{44} stabilized the meta II (MII) photoproduct relative to forms MI and MIII and did not interact measurably with the apoprotein opsin. However, several differences between p^{44} and its parent protein were found: (i) p^{44} binds to nonphosphorylated MII with a much lower affinity (K_D = 0.24 μM) than to phosphorylated MII (P-MII) (K_D = 12 nM); arrestin binds only to P-MII (K_D = 20 nM); (ii) p^{44} interacted also with truncated MII (^{329}G-Rho MII), which lacked the sites of phosphorylation; (iii) with both MII and P-MII, the activation energy of complex formation with p^{44} was lower than that found for arrestin (70 kJ/mol instead of 140 kJ/mol); and (iv) $InsP_6$ inhibited poorly the interaction between p^{44} and P-MII, but it strongly inhibited the interaction between arrestin and P-MII. Extrapolation of the measured on-rates to physiological conditions yielded reaction times for the binding of p^{44} to activated rhodopsin. The data suggest that the splice variant, p^{44}, and its parent protein, arrestin, play different roles in phototransduction. The physiological significance of these differences remains to be determined.

In retinal rod photoreceptor cells, inactivation of photolyzed rhodopsin proceeds by a two-step mechanism comprising phosphorylation by a specialized receptor kinase and subsequent binding of the 48 kDa regulatory protein arrestin [reviewed by Polans *et al.* (1996) and Palczewski (1994)]. This process depends on the strict preference of arrestin for phosphorylated photolyzed rhodopsin. However, a recently identified splice variant of arrestin, termed p^{44}, binds both phosphorylated and nonphosphorylated forms of the receptor (Palczewski *et al.*, 1994). p^{44}, in which the last exon encoding the C-terminal 35 amino acids is replaced by an alternative exon encoding a single Ala, has been immunolocalized to rod outer segments (ROS)[1] (Smith *et al.*, 1994). In reconstitution assays composed of nonphosphorylated rhodopsin and phototransduction proteins, activation of the effector enzyme, a cGMP phosphodiesterase, was inhibited by p^{44} (Palczewski *et al.*, 1994). Addition of p^{44} to ROS preparations shortened the duration of the heat response due to cGMP hydrolysis by the effector enzyme (Langlois *et al.*, 1996), interpreted as a competitive inhibition of the photoactivated rhodopsin.

In this study, we investigated the kinetics, activation energy, and stability of the complex between p^{44} and different forms of rhodopsin. Like arrestin, p^{44} bound specifically to the MII form of active rhodopsin. However, p^{44} differed from arrestin by binding to nonphosphorylated photolyzed rhodopsin and to a C-terminally truncated form of rhodopsin, which lacks the potential sites of phosphorylation. On the basis of these and other observations, we propose a mechanism by which p^{44} and arrestin form complexes with different forms of rhodopsin and discuss the consequences of these interactions to the inactivation processes that occur during phototransduction.

MATERIALS AND METHODS

Isolation of Bovine Rod Outer Segments (ROS). Bovine ROS were prepared from fresh, dark-adapted retinas obtained from a local slaughterhouse by means of a discontinuous sucrose gradient method (Papermaster, 1982). Retinas were dissected, and ROS were isolated, under dim red illumination. All subsequent procedures were performed at 0−5 °C.

Purification of Rhodopsin. Rhodopsin was prepared by removing the soluble and membrane-associated proteins from native ROS membranes by repetitive washes with a low ionic strength buffer (Kühn, 1982). All steps were performed under dim red illumination. The membrane suspension was stored at −80 °C until use.

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[1] Abbreviations: BTP, bis-tris-propane; $InsP_6$, inositol-hexakisphosphate; MI, metarhodopsin I; MII, metarhodopsin II; MIII, metarhodopsin III; P-MII, phosphorylated MII; ROS, rod outer segments.

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Purification of Phosphorylated Opsin. Phosphorylated opsin was prepared from washed disk membranes as described previously (Wilden & Kühn, 1982).

Purification of Phosphorylated Rhodopsin. Phosphorylated rhodopsin was prepared by the regeneration of phosphorylated opsin with 11-*cis*-retinal (Hofmann *et al.*, 1992). The complete characterization of phosphorylated species of rhodopsin prepared according to this protocol was reported by Oghuro *et al.* (1993). Reconstitution systems of rhodopsin phosphorylation yields predominantly monophosphorylated species (an average stoichiometry of ~1.5 phosphates per rhodopsin on several Ser and Thr residues) (Ohguro et al., 1993). Phosphorylated opsin was suspended in 10 mM BTP, pH 7.5, containing 100 mM NaCl. A 3-fold molar excess of 11-*cis*-retinal was added in the dark to the sample, followed by incubation for 1 h at room temperature, and then overnight at 4 °C. After regeneration, phosphorylated membranes were centrifuged at 45000*g* for 20 min, washed five times with 10 mM BTP, pH 7.5, containing 100 mM NaCl to remove excess 11-*cis*-retinal, and stored at −80 °C. 11-*cis*-Retinal was a gift from Dr. D. S. Kliger and from the National Eye Institute.

Purification of Arrestin. Arrestin was purified from frozen, dark-adapted bovine retinas as described by Palczewski *and* Hargrave (1991).

Purification of p^{44}. p^{44} was purified as described by Palczewski *et al*. (1994), with modifications. All extraction steps were performed under dim red illumination at 4 °C. Freshly prepared bovine ROS (obtained from 300 bovine retinas) were suspended and homogenized (teflon/glass) in 50 mL of a hypotonic buffer (10 mM BTP, pH 8.0, containing 1 mM benzamidine). ROS membranes were collected by centrifugation at 47000*g* for 30 min, and the supernatant was discarded. The pellet was resuspended and homogenized (glass/glass) in 25 mL of 10 mM BTP, pH 8.0, containing 1 mM benzamidine, 500 mM NaCl, and 15 mM $InsP_6$. The supernatant containing p^{44} was separated from the membranes by centrifugation at 47000*g* for 20 min. This p^{44} extraction was repeated two times. SDS−PAGE analysis revealed that p^{44} was concentrated in the first extract (~80% of the total available p^{44}). The p^{44} extracts were pooled and dialyzed against 3 L of 10 mM BTP, pH 7.5, containing 1 mM benzamidine. p^{44} was purified on an HPLC TSK gel-Heparin-5PW column (TosoHaas, GmbH, stainless steel column, 0.75 × 7.5 cm, 10 μm particle size) equilibrated with 10 mM BTP, pH 8.4, using an FPLC (Pharmacia Biotech, Inc., Piscataway, NJ). The column was washed with the same buffer at a flow rate of 0.5 mL/min until the absorbance at 280 nm dropped below 0.02. p^{44} was eluted with a gradient from 0 to 600 mM NaCl in the same buffer and with the same flow rate. Fractions of 0.5 mL were collected and analyzed by SDS−PAGE. Purified p^{44} (eluted at ~450 mM NaCl) was dialyzed against 10 mM BTP, pH 7.5, containing 100 mM NaCl and concentrated on a Centricon 30 (Amicon, Beverly, MA). Approximately 0.3 mg of homogeneous p^{44} was obtained from ROS prepared from 300 retinas.

Preparation of C-Terminally Truncated Rhodopsin (^{329}G-Rho). Membrane-bound rhodopsin was digested with endoproteinase Asp-N, as described previously (Palczewski *et al.*, 1991b), except that the incubation was carried out in buffered sucrose solutions. The addition of sucrose prevents membrane aggregation, which was observed in preparations containing the truncated form of membrane-bound rhodopsin. Briefly, ROS or urea-treated ROS membranes were incubated with endopeptidase Asp-N at 6000:1 (w:w, rhodopsin:Asp-N) in 10 mM Tris-Cl, pH 7.5, containing 0.3 M sucrose, at room temperature for 16 h in the dark. The reaction was stopped by adding 1 mM DTT and 1 mM EDTA, followed by centrifugation at 30000*g* for 20 min at 4 °C. Membranes were treated with buffered urea solutions (50 mM Tris, 5 M urea, 5 mM EDTA, pH 7.5) to extract bound C-terminal peptides and pelleted by centrifugation at 30000*g* for 30 min. Pelleted membranes were washed three times with 50 mM Tris-Cl, pH 7.5, containing 0.3 M sucrose and resuspended in the same buffered sucrose solution. Samples were stored at −80 °C. Conversion of rhodopsin to the truncated ^{329}G-Rho form lacking its C-terminal sequence 330−348 (yield > 85%) was confirmed in aliquots of the preparation by SDS−PAGE, according to its decrease in molecular weight, as described previously, and by the decrease in ^{32}P incorporation by rhodopsin kinase (Palczewski *et al.*, 1991b).

Preparation of Recombinant p^{44}. *Nco*I and *Hin*dIII restriction sites were added to the 5′- and 3′-ends, respectively, of the p^{44} cDNA by PCR (Smith *et al.*, 1994). This cDNA was then cloned into the appropriate sites of the pBluBacIII and pBacHisC shuttle plasmids (Invitrogen). High Five insect cells (Invitrogen) were infected with the recombinant virus pVL1392 baculovirus carrying the p^{44} insert. Cells were harvested four days after the infection and collected by centrifugation at 2000*g* for 5 min. Five to seven milliliters of pelleted cells were typically used for the purification. Cells were homogenized (glass/glass) with 10 mM BTP, pH 7.5, containing 200 mM NaCl and 1 mM benzamidine at a ratio of 5 mL of buffer per 1 mL of pelleted cells. Homogenates were pooled and centrifuged at 145000*g* for 30 min at 4 °C. The final supernatant was diluted 1:1 with 10 mM BTP, pH 7.5, containing 1 mM benzamidine followed by centrifugation at 145000*g* for 20 min at 4 °C. The extracted proteins were loaded on to a DEAE-cellulose column (1.5 × 8 cm) equilibrated with 10 mM BTP, pH 7.5, containing 100 mM NaCl (saline-BTP) at a flow rate of 0.5 mL/min. Eluate was directly loaded onto a heparin-Sepharose column (1 × 5 cm) equilibrated with the same buffer. After the material was loaded, both columns were washed with 15 mL of saline-BTP, and the DEAE-cellulose column was detached. The heparin-Sepharose column was washed consecutively with saline-BTP (until the absorbance at 280 nm dropped below 0.1) and two column volumes of 275 mM NaCl in 10 mM BTP, pH 7.5. Next, the column was equilibrated with 10 mM BTP, pH 7.5, containing 200 mM NaCl, and p^{44} was eluted with a gradient from 0 to 20 mM $InsP_6$ in 10 mM BTP, pH 7.5, containing 200 mM NaCl. Fractions containing p^{44} were identified by immunoblot using a C-terminal-specific antibody (C88) (Smith *et al.*, 1994). Pooled fractions containing p^{44} from DEAE-cellulose were dialyzed against 10 mM BTP, pH 7.5, containing 1 mM benzamidine (3 L) and loaded onto an HPLC hydroxyapatite column (Pentax, SH-0710M) at a flow rate of 1 mL/min. After washing the column with 10 mM BTP, pH 7.5, a 0 to 250 mM phosphate gradient in 10 mM BTP, pH 7.5, containing 1 mM benzamidine, was generated in 45 min at a flow rate of 0.8 mL/min. Fractions with p^{44} (protein and immunoreactivity profiles) were pooled and dialyzed overnight against 10 mM BTP, pH 7.5, containing 1 mM benzamidine. Dialyzed fractions from hydroxyapatite con-

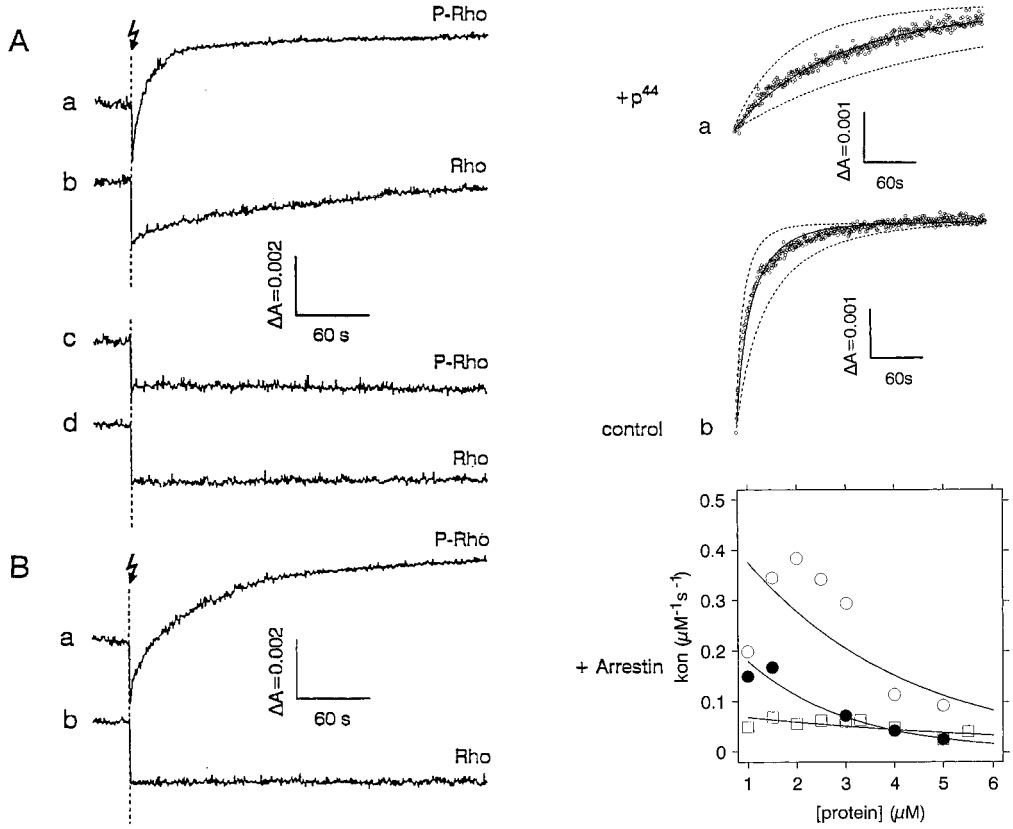

FIGURE 1: Flash-induced formation of extra MII in a suspension of washed disk membranes. The signals represent the absorbance change at 380 nm minus the absorbance change at 417 nm (see Materials and Methods). (A) Extra MII formation in phosphorylated (a) and nonphosphorylated (b) washed ROS membranes in the presence of p^{44}. In all measurements, the final concentrations in the samples were 15 μM Rho (nonphosphorylated washed ROS membranes) or 15 μM P-Rho (phosphorylated washed ROS membranes) and 3 μM p^{44} in 100 mM BTP, pH 8.0, at 4 °C. Total volume was 200 mL, cuvette path length was 2 mm, and the flash photolyzed 12% of rhodopsin. Control with P-Rho (c) and Rho (d) alone. The abrupt decrease after the flash is due to MI formation. (B) Extra MII formation in phosphorylated (a) and nonphosphorylated (b) washed ROS membranes under identical conditions as in panel A, but in the presence of 3 μM purified arrestin instead of p^{44}. (C) Bimolecular reaction fit of extra MII formation in nonphosphorylated (a) and phosphorylated (b) ROS membranes in the presence of 5 μM purified p^{44}. Solid lines represent best fit according to the equation described in the Appendix. Dotted lines are the kinetics with twice or half the on-rate assumed. (D) Plot of the on-rate (from fit as shown in Figure 1C). Symbols are (○), P-MII-p^{44}; (●), MII-p^{44}; and (□), P-MII-arrestin.

taining p^{44} were loaded onto an HPLC TSKgel-Heparin column at a flow rate of 1 mL/min, washed with 10 mM BTP, pH 7.5, and followed by a 0 to 200 mM phosphate gradient in the same buffer in 60 min at a flow rate of 0.4 mL/min. Fractions were analyzed by SDS−PAGE and blotted for immunoreactivity.

SDS−PAGE. Electrophoresis was performed according to Laemmli (1970) using 12% acrylamide gels in a Hoefer or a Bio-Rad minigel apparatus.

Protein Determinations. The concentration of rhodopsin and phosphorylated rhodopsin was determined spectrophotometrically at 498 nm as previously described (Wald & Brown, 1953). Purified arrestin and p^{44} were quantified spectrophotometrically at 278 nm, assuming a molar absorption coefficient of $E_{0.1\%} = 0.638$ (Palczewski *et al.*, 1992) and a molecular mass of 45 300 Da for arrestin or 41 200 Da for p^{44} (Palczewski *et al.*, 1994).

Spectrophotometrical Analysis. A two-wavelength spectrophotometer (Shimadzu UV300) was used to measure the differences in absorption at 380 (photoproduct of MII) and at 417 nm after a flash filtered to 500 ± 20 nm. The isosbestic point between MI and MII at 417 nm was used to subtract light-scattering artifacts from the absorption signal (Schleicher *et al.*, 1989). When rhodopsin, in its native disk membrane, is cooled to temperatures at which the equilibrium is on the MI side (below 5 °C, pH 8.0) (Parkes & Liebman, 1984), binding of a protein to MII causes an increase of MII (extra MII). Extra MII provides a kinetic and stoichiometric measure for the complex between active rhodopsin and the interactive proteins [reviewed by Hofmann (1985)]. The mole fraction of activated rhodopsin in the sample was 12%.

RESULTS

p^{44} Enhanced the Formation of Metarhodopsin II (MII) for Pre-[2] *and Nonphosphorylated ROS Membranes.* The binding of a protein to photoactivated rhodopsin either enhances the formation of the MII photoproduct after a flash of light (for example, G_t or arrestin; Emeis *et al.*, 1982; Schleicher *et al.*, 1989) or, if the interaction is not specific for MII, competes with a stabilizing protein (for example, the competition between rhodopsin kinase and G_t, Pulvermüller *et al.*, 1993). The enhanced MII formation is shown in Figure 1A for prephosphorylated membranes (P-Rho, trace a) and nonphosphorylated ROS membranes (Rho, trace b) in the presence of p^{44}. Controls without p^{44} are shown as traces c and d. The data demonstrate that p^{44}, like its parent

[2] Prephosphorylated rhodopsin is obtained by phosphorylation of photolyzed rhodopsin by rhodopsin kinase and regeneration with 11-*cis*-retinal.

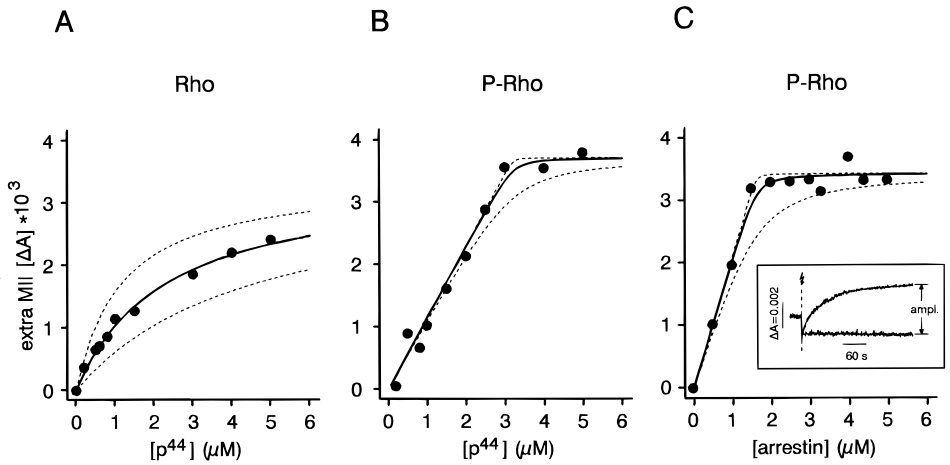

FIGURE 2: Titration of extra MII formation by p^{44} or arrestin. (A) Saturation of the p^{44}-dependent extra MII formation in ROS membranes as a function of the p^{44} concentration. The plot shows final amplitudes of the p^{44}-dependent component of the difference absorption signals from a first flash on a fresh sample. The best-fit curve (solid line) to the data yields a dissociation constant for the Rho*-p^{44} interaction of $K_D = 0.24$ μM. Upper and lower dashed lines represent curves for K_Ds of 0.12 and 0.48 μM, respectively. (B) As in panel A, but phosphorylated ROS membranes. The best-fit curve (solid line) yields in this case $K_D = 0.012$ μM. Upper and lower dashed lines represent curves for K_Ds of 0.001 and 0.1 μM, respectively. (C) Saturation of the arrestin-dependent extra MII in phosphorylated ROS membranes. Best-fit curve (solid line) gives $K_D = 0.02$ μM. Upper and lower dashed lines represent curves for K_Ds of 0.002 and 0.2 mM, respectively. The experiment was otherwise performed as described in the legend for Figure 1.

protein arrestin (Figure 1B), can stabilize P-MII. In contrast to arrestin, p^{44} also stabilized MII. The results suggest that the C-terminal region of arrestin is not essential for the MII stabilization.

A Bimolecular Reaction Scheme Fits the p^{44} Binding. The absorption changes that represent the enhanced formation of MII (or P-MII) can be evaluated by a kinetic scheme for a bimolecular reaction:

$$(\text{MII-X}) = k_{on}(\text{MII})(\text{X})$$

where X is the interacting protein and k_{on} is the bimolecular rate constant in inverse micromolar seconds. For a wide range of p^{44} concentrations, the binding to MII and P-MII validated the bimolecular fit, suggesting a kinetically homogeneous pool of this protein. For example, there was no indication of a variable distribution of the protein between a membrane-bound form and a soluble form (Figure 1C). The centrifugation of rhodopsin and p^{44} mixtures showed that, under these experimental conditions, the splice variant of arrestin was soluble (data not shown).

Applying the fit procedure to the whole set of data yielded the on-rates of the reactions of P-MII and MII with p^{44} and of arrestin with P-MII. For arrestin up to 5 μM, the k_{on} decreased only slightly with increasing concentration (Figure 1D). For p^{44} and both P-MII and MII, the k_{on} was ~3 times faster at low concentrations. Although we do not know the cause for this phenomenon, a possible explanation is the aggregation of p^{44} in solution, as observed using gel filtration (data not shown). At 1 μM, p^{44} binds ~5 times faster to MII and ~8 times faster to P-MII than arrestin to P-MII.

To determine the dissociation constants (K_D) of the interactions between p^{44} with P-MII or MII and arrestin with P-MII, we employed the procedure introduced by Schleicher et al. (1989). At a constant concentration of photolyzed rhodopsin and varying p^{44} concentration, the extra MII signal amplitude increased and exhibited saturation, yielding the K_D for the complex (Figure 2). To fit the data, the mass action law was used with the conservation of the total amount of MII and p^{44} (Figure 2A) or P-MII and p^{44} or arrestin (Figure 2, panels B and C). The formula used is given in Schleicher et al. (1989). For MII and p^{44} interaction, the K_D was 0.24 μM (Figure 2A), and for the MII-P and p^{44} (Figure 2B), the K_D was 0.012 μM. In a control experiment, a $K_D = 0.02$ μM was found for arrestin and MII-P, similar to the value reported by Schleicher et al. (1989) (Figure 2C).

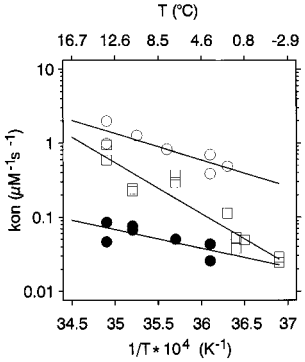

FIGURE 3: Temperature dependence and Arrhenius plots of the rate of extra MII formation by p^{44} or arrestin. Flash-induced formation of extra MII was measured as a function of temperature at pH 8.0, with phosphorylated photolyzed rhodopsin or nonphosphorylated photolyzed rhodopsin, and in the presence of 2 μM p^{44} or 2 μM arrestin. The experiment was otherwise performed as described in the legend for Figure 1. The signals were analyzed by a bimolecular reaction fit (see Appendix). For the Arrhenius representation, the on-rate (k_{on}) is plotted as a function of the inverse absolute temperature. Symbols are (○), P-MII-p^{44}; (●), MII-p^{44}; and (□), P-MII-arrestin.

Activation Energy of p^{44} Complex with MII Is Different from Arrestin. The kinetics of complex formation, as reflected in the MII stabilization, were obtained within a limited range of temperatures (0−14 °C). The higher temperatures lead to a significant spontaneous formation of MII, obscuring the accuracy of the measurements. For the permissible temperatures, the bimolecular fit procedure (Figure 1B) was employed, and the k_{on} values were plotted versus temperature for the different complexes (MII−p^{44}, P-MII−p^{44}, and P-MII−arrestin; Figure 3). From the equation

$$k_{on} \approx \exp(-E_a/kT)$$

the activation energy was obtained for the formation of the respective complexes. It is important to note, however, that the activation energy obtained does not incorporate the enthalpy of MII formation. The E_a for the P-MII−arrestin complex was 140 kJ/mol, lower than the 165 kJ/mol value obtained by Schleicher et al. (1989). Among several possibilities for this discrepancy, it is important to note that, in the previous study, pseudo-first-order rates were employed instead of the correct bimolecular on-rates and that different salt conditions in the reaction mixture were used. The high E_a is necessary for arrestin to adopt the interactive conformation that involves the possible movement of the C-terminus (Schleicher *et al.*, 1989; Palczewski *et al.*, 1991c). Interestingly, the E_a for the complexes of p^{44} with both MII and P-MII was significantly lower (~70 kJ/mol). For p^{44}, such changes in conformation are unnecessary due to the absence of the C-terminus.

Hydroxylamine Dissociates the p^{44}−MII Complex. In contrast to MI and MIII, the MII state of rhodopsin is distinguished by its sensitivity to hydroxylamine (Kamps & Hofmann, 1986; Ernst *et al.*, 1995). At millimolar concentrations of this nucleophilic compound, the MII complex with G_t (Hofmann et al., 1983) or arrestin (Hofmann et al., 1992) dissociates within tens of seconds. The result shown in Figure 4 is based on the fact that all p^{44} molecules remain bound to P-MII after a flash at a subsaturating amount of p^{44}. Thus, the P-MII from a second and third flash does not find free p^{44} to bind, and no extra MII is observed (as seen in the control in Figure 4B). In the presence of hydroxylamine, however, extra MII formation recovered after each flash (Figure 4A), because the pool of free p^{44} is restored after each flash due to dissociation of the complex. The experiment confirms the specificity of p^{44} for the deprotonated Schiff base form of rhodopsin, MII, and shows that the complex remains stable during the whole lifetime of the photoproduct.

p^{44} Stabilizes the Meta II State of Truncated Rhodopsin. Because arrestin binds only to P-MII, whereas p^{44} binds to both P-MII and MII, we investigated the ability of p^{44} to bind to C-terminally truncated MII, which lacks the phosphorylation region. The experiment in Figure 5A demonstrates that p^{44} stabilized the ^{329}G-MII state of rhodopsin, at least to the same extent as for MII. Thus, rhodopsin's C-terminus is not required for p^{44} binding. Arrestin did not bind the truncated rhodopsin (Figure 5B).

$InsP_6$ Weakly Inhibits p^{44}−MII Interaction. $InsP_6$ has been shown to inhibit the interaction of arrestin with phosphorylated active rhodopsin (Palczewski *et al.*, 1991a). Figure 6 shows that $InsP_6$ inhibited the interaction between p^{44} and both P-MII and MII much less than between arrestin and P-MII. The IC_{50} was ~2000 μM for p^{44} and only 20 μM for arrestin.

Expression of p^{44} in Insect Cells. p^{44} was expressed in insect cells using baculovirus carrying a p^{44} DNA insert. Recombinant p^{44} was purified from HighFive insect cells with a reasonable yield of 0.1 mg from 20 150 mm plates (Figure 7A). Purified recombinant p^{44} displayed a binding profile undistinguishable from native protein (for example, Figure 7B). A similar purification scheme might be employed with some modification in other expression systems for native and mutated forms of p^{44}.

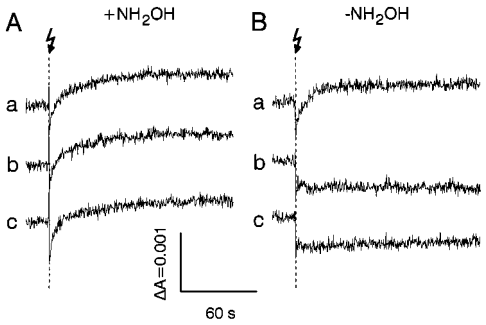

FIGURE 4: Recovery of the p^{44}-dependent extra MII formation by hydroxylamine. (A) Extra MII formation in phosphorylated washed ROS membranes in the presence of p^{44} and 2 mM hydroxylamine. (B) Extra MII formation without hydroxylamine. In each panel, record (a) is from the first and record (b) from the second flash, applied at a time interval <3 min. A third flash, record c in each panel, was applied after 5 min; note the difference in the recovery of the signals in panel A vs B. The conditions of the experiment were as described in the legend for Figure 1.

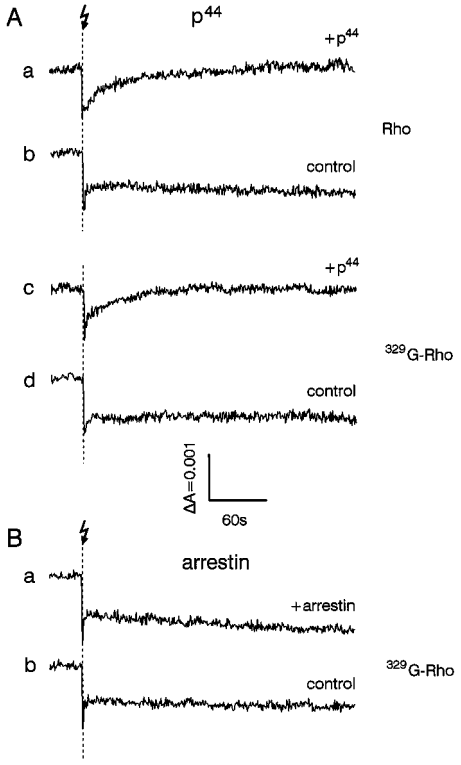

FIGURE 5: Binding of p^{44} or arrestin to C-terminally truncated rhodopsin. (A) Extra MII formation in nonphosphorylated (a) and endopeptidase Asp-N digested (c) washed ROS membranes, both in the presence of p^{44}. In all measurements, the final concentrations in the samples were 15 μM rhodopsin or 15 μM ^{329}G-Rho and 1 μM p^{44} in 100 mM BTP, pH 8.0, at 4 °C. Control with rhodopsin (b) and ^{329}G-Rho (d) alone. The abrupt decrease after the flash is due to MI formation. (B) Extra MII formation in endopeptidase Asp-N, digested washed ROS membranes (a) under identical conditions as in panel A, but in the presence of 1 μM purified arrestin instead of p^{44}. Control with ^{329}G-Rho alone (b). The experiment was otherwise performed as described in the legend for Figure 1.

DISCUSSION

p^{44} is a rod outer segment-specific, membrane-associated splice variant form of arrestin, whose function is not yet

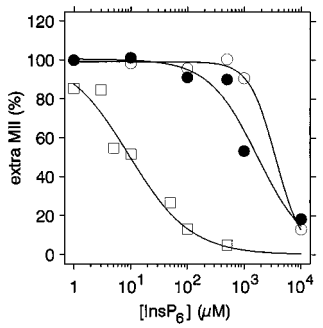

FIGURE 6: Inhibition of the interaction between p^{44} and rhodopsin by $InsP_6$. Flash-induced formation of MII or P-MII, as in Figure 1. Shown is the level of extra MII formed by interaction with p^{44} or arrestin minus the control MII as a function of ligand concentration. Symbols are (○) the effect of $InsP_6$ on P-MII-p^{44} interaction, IC_{50} = 3600 μM, the Hill coefficient was 1.9; (●) the effect of $InsP_6$ on the MII-p^{44} interaction, IC_{50} = 1700 μM, the Hill coefficient was 1.0; and (□) the effect of $InsP_6$ on P-MII-arrestin interaction, IC_{50} = 18 μM, the Hill coefficient was 1.1. The lines through the data are computer fits with hyperbolic functions.

clearly defined. p^{44} is a natural variant in which the last 35 amino acids are replaced by a single Ala, making it an attractive protein for studing the role of the divergent C-terminal region of mammalian arrestins.

Specificity of Arrestins Binding to Rhodopsins. We chose a time-resolved spectroscopy, the "extra MII" stabilization assay, to study arrestin and p^{44} interaction with MII and P-MII (Schleicher *et al.*, 1989). The binding of arrestins was induced by a short flash of light that activated a fraction of the total pool of rhodopsin. The assay does not depend on the association of the interacting protein with the membrane before photoexcitation, and, at 5 μM, p^{44} stabilized P-MII almost completely. Thus, MII is the only intermediate to which p^{44} binds. There is no significant interaction with any of the tautomeric forms, MI and MIII, metarhodopsins which bind the retinal chromophore in its *trans*-configuration via a protonated Schiff base bond. Thus, the conformational changes of rhodopsin that occur during the transition to MII are necessary for p^{44} binding. Similar findings were also found for arrestin (Schleicher *et al.*, 1989).

The phosphorylated C-terminus of MII is required for arrestin to bind to the receptor. If the rhodopsin C-terminus is removed or not phosphorylated, arrestin does not recognize the remaining sites of MII. The phosphorylated C-terminus of rhodopsin may displace the acidic C- terminal region in arrestin, inducing an active conformation of arrestin and securing tight binding to MII. The large activation energy of the interaction suggests some major changes in the conformation (Schleicher *et al.*, 1989). This is also consistent with earlier findings that arrestin bound to P-MII assumed a binding conformation with its C-terminus accessible to proteolysis (Palczewski *et al.*, 1991d) and that synthetic phosphopeptides encompassing the C-terminus of rhodopsin induced the binding of arrestin to photolyzed, nonphosphorylated rhodopsin (Puig *et al.*, 1995). In contrast to arrestin, p^{44} requires a smaller activation energy (Figure 3) to bind and stabilize P-MII. p^{44} also binds to MII (Palczewski *et al.*, 1994; this study) and ^{329}G-MII which lacks the C-terminus phosphorylation region. However, p^{44} binds to MII more slowly and much more weakly than to P-MII. Without phosphorylation of the receptor, the complex has a ~10-fold enhanced off-rate, suggesting that MII is less capable of retaining the bound protein. This shows that the

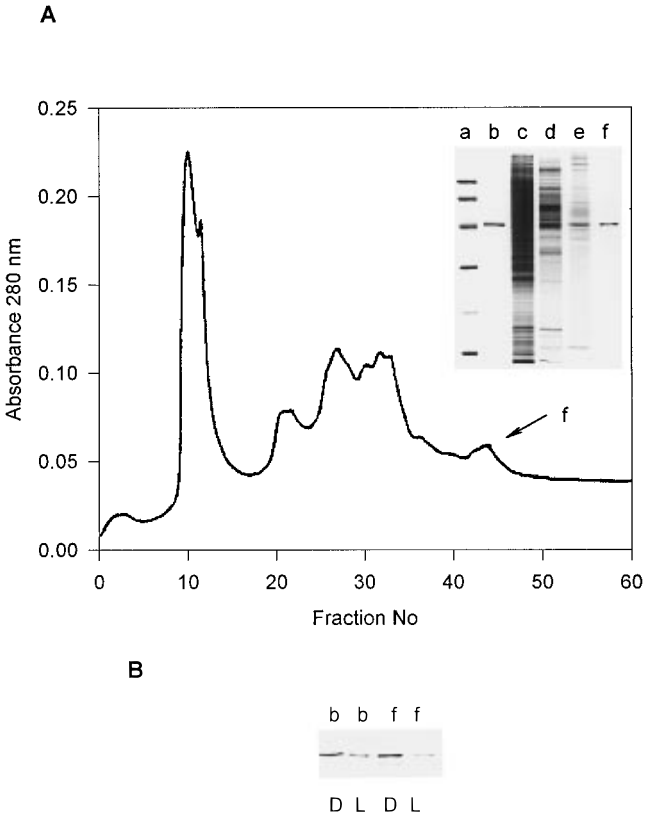

FIGURE 7: Purification and functional assay of recombinant p^{44}. (A) The TSK-heparin chromatographic profile of partially purified recombinant p^{44}. p^{44} was extracted from infected HighFive insect cells and partially purified by employing a sequence of chromatographic steps: DEAE-cellulose, Heparin-Sepharose, and hydroxyapatite, as described in Materials and Methods. The absorption was monitored at 280 nm during the elution with 0−200 mM potassium phosphate in 10 mM BTP, pH 7.5, over 60 min at a flow rate of 0.4 mL/min. A small absorption peak at ~43 min (depicted as f) represents eluted p^{44}. (Inset) Protein profiles during different steps of purification using SDS−PAGE. The lanes represent (a) molecular weight markers (from the top in kDa: 92, 67, 43, 30, 20, and 14); (b) purified p^{44} isolated from ROS; (c) HighFive cell extract; (d) the pooled fractions containing p^{44} obtained from DEAE-cellulose/Heparin-Sepharose; (e) the pooled fractions containing p^{44} obtained from hydroxyapatite; and (f) purified recombinant p^{44} eluted from the TSK-heparin column. (B) The binding of native (as in lane b in the inset to panel A) and recombinant p^{44} (as in lane f in the inset to panel A) to phosphorylated, photolyzed rhodopsin. Under dim red illumination, phosphorylated rhodopsin (25 μM) was mixed with purified bovine p^{44} (5 μM, depicted as b in the inset to panel A) or recombinant p^{44} (5 μM, depicted as f in the inset to panel A) in 10 mM BTP, pH 7.5, containing 100 mM NaCl. The samples were incubated in the dark (D) or under white illumination (L) at room temperature. p^{44}·MII complex was centrifuged at 30000*g* for 3 min. The resulting supernatant was mixed with 1% SDS, subjected to SDS−PAGE, and transferred to Immobilon. p^{44} was visualized using C88 polyclonal antibody (Smith *et al.*, 1994).

phosphorylated residues on P-MII are not only involved in the switching process but are also required for permanent tight binding of p^{44} or arrestin. The work with arrestin mutants has led to a similar mechanistic model in which the C-terminus of arrestin is involved in the conformational switch but is not involved in binding to rhodopsin (Gurevich & Benovic, 1997 and references therein).

Inhibition by $InsP_6$. The reduced competition of $InsP_6$ with p^{44} for active rhodopsin, as compared with arrestin (Figure 6), can be explained by the same line of reasoning. $InsP_6$ possibly mimics the phosphate environment on the phosphorylated C-terminal extension of active rhodopsin (Pal-

czewski et al., 1991c). Thus, it can block the interaction with phosphorylated, photolyzed rhodopsin because it prevents the association of arrestin with the phosphorylated region of rhodopsin. With p^{44}, however, the competition of $InsP_6$ with MII or P-MII is significantly less effective.

Role in Phototransduction. The rate of binding of p^{44} to MII was obtained from the kinetic data of Figure 1 and Figure 3. The highest measurable on-rate of the binding was 0.08 μM^{-1} s^{-1} (Figure 3; 14 °C, 2 μM p^{44}), which was estimated to be 0.28 μM^{-1} s^{-1} at 34 °C. The calculated reaction rate at 30 μM concentration of p^{44} in the rod outer segments (Palczewski *et al.*, 1994), taking into account the concentration dependence of the on-rate (represented by a correction factor *K*), is

$$k = K(30)(0.28) = K8.4\ s^{-1}$$

The factor *K* was obtained from Figure 1D, assuming that the correction is temperature independent and valid for cellular conditions. For $K \geq 0.15$, the time of the MII−p44 complex formation is ≤ 0.8 s^{-1}, and an estimated time of complex formation between a newly formed MII and p^{44} is

$$T = 0.5-1\ s$$

The spontaneous off-rate was estimated from the on-rate (0.28 μM^{-1} s^{-1}) and the dissociation constant

$$k_{off} = k_{on}K_D$$

which yields $k_{off} = (0.28\ \mu M^{-1}\ s^{-1} \cdot 0.24\ \mu M) = 0.07\ s^{-1}$ or ~15 s, long enough to keep the MII−p44 complex stable for a time much longer than the rod response.

Rhodopsin kinase binds ~5 times faster than p^{44} to MII (1 μM^{-1} s^{-1}; Pulvermüller *et al.*, 1993). The competition with kinase is slowed by the interference of G_t activation. Nonetheless, the breaks between the engagements of the MII in MII−G_t complexes are long enough to allow the kinase to bind with some delay and to phosphorylate photolyzed rhodopsin (Felber *et al.*, 1996). Phosphorylation of MII enhances the affinity of both p^{44} and arrestin. As long as free arrestin is available, it will usually win the kinetic competition due to its much higher concentration. However, it should be noted that p^{44} used under the conditions of our measurements behaves as a soluble protein, although it may be membrane associated *in vivo* (Smith *et al.*, 1994). Thus, *in vivo*, there may be differences in concentration of this protein within the cell. The recent generation of mice lacking the arrestin gene opens the possibility for studying the role of individual forms of arrestins in phototransduction *in vivo* without interference by other splice forms.

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APPENDIX

Extra MII is described by the reaction

$$MI + X \overset{K_1}{\rightleftharpoons} MII + X \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} MIIX \quad (1)$$

where MI and MII are metarhodopsin I and II. X denotes a protein that binds specifically to MII, in this study p^{44} or arrestin.

The equilibria obey the equations

$$K_1 = \frac{[MII]}{[MI]} \quad (2)$$

$$\frac{k_{on}}{k_{off}} = \frac{[MIIX]}{[MII][X]} \quad (3)$$

where [MI], [MII], and [X] represent the molar concentration of MI, MII, and X, respectively. K_1 is the equilibrium constant of MI/MII and calculated after Parkes and Liebman (1989), k_{on} the on-rate, and k_{off} the off-rate of the MIIX complex.

From the conservation of mass,

$$[R^*]_{tot} = [MI] + [MII] + [MIIX] \quad (4)$$

$$[X]_{tot} = [X] + [MIIX] \quad (5)$$

$[R^*]_{tot}$ and $[X]_{tot}$ are the total concentration of photolyzed rhodopsin and X protein. By use of eqs 2 and 4, [MII] can be expressed as

$$[MII] = \frac{K_1}{K_1 + 1}([R^*]_{tot} - [MIIX]) \quad (6)$$

The rate for complex formation is

$$\frac{d[MIIX]}{dt} = k_{on}\frac{K_1}{K_1 + 1}([R^*]_{tot} - [MIIX])([X_{tot}] - [MIIX]) - k_{off}[MIIX] \quad (7)$$

at equilibrium

$$k_{on}\frac{K_1}{K_1 + 1}([R^*]_{tot} - [MIIX]_\infty)([X]_{tot} - [MIIX]_\infty) = k_{off}[MIIX]_\infty \quad (8)$$

$[MIIX]_\infty$ represents the molar concentration of the complex after an infinitely long time and was determined from the K_D.

With eq 8, eq 7 is integrated to give

$$\ln\frac{([MIIX]_\infty - [MIIX])([R^*]_{tot}[X]_{tot})}{[MIIX]_\infty([R^*]_{tot}[X]_{tot} - [MIIX]_\infty[MIIX])} = \frac{[MIIX]_\infty^2 - [R^*]_{tot}[X]_{tot}}{[MIIX]_\infty}k_{on}\frac{K_1}{K_1 + 1}\,t \quad (9)$$

This can be rearranged to give

$$[MIIX] = \frac{[MIIX]_\infty[R^*]_{tot}[X]_{tot}\left(\exp\left(Zk_{on}\frac{K_1}{K_1 + 1}t\right) - 1\right)}{[MIIX]_\infty^2\left(\exp\left(Zk_{on}\frac{K_1}{K_1 + 1}\,t\right) - [R^*]_{tot}[X]_{tot}\right)} \quad (10)$$

where

$$Z = \frac{[\mathrm{MIIX}]_\infty^2 - [\mathrm{R}^*]_{tot}[\mathrm{X}]_{tot}}{[\mathrm{MIIX}]_\infty}$$

Equation 10 was used to fit the extra MII data. A scaling factor (σ) relates the measured values to corresponding concentration units.

$$\Delta A = \sigma[\mathrm{MIIX}] \quad (11)$$

where ΔA are the absorption change at 380 nm.

In all fits, $[\mathrm{MIIX}]_\infty$, $[\mathrm{R}^*]_{tot}$, $[\mathrm{R}^*]_{tot}$, $[\mathrm{X}]_{tot}$, and K_1 were fixed, and k_{on} and σ were allowed to vary.

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