Constitutive Activation of Opsin: Interaction of Mutants with Rhodopsin Kinase and Arrestin†

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ABSTRACT: Mutation of Gly^{90}, Glu^{113}, Ala^{292}, and Lys^{296} in the visual pigment rhodopsin constitutively activates the protein for activation of the G protein transducin. Three of these mutations have been shown to cause two different human diseases. Mutation of Gly^{90} and Ala^{292} results in complete night blindness, and mutation of Lys^{296} results in the degenerative disease retinitis pigmentosa. We show here that the mutants not only constitutively activate transducin but are also constitutively activated for phosphorylation by rhodopsin kinase. In addition, the phosphorylated mutants are shown to bind tightly to the inhibitory protein arrestin in a reaction that quenches the activity toward transducin. Thus the same mutations that result in constitutive activation of transducin also result in constitutive phosphorylation by rhodopsin kinase and binding of arrestin to inhibit the activity. This implies that the same conformational change may be responsible for activation of transducin and rhodopsin kinase. It also suggests that degeneration of photoreceptor cells in retinitis pigmentosa results indirectly from the activated state of the receptor, perhaps as a consequence of phosphorylation and persistent binding of arrestin.

The visual pigment rhodopsin is a prototype member of the large family of G protein-linked receptors (Dohlman et al., 1991; Oprian, 1992). Rhodopsin is composed of an apoprotein opsin and an 11-*cis*-retinal chromophore covalently attached to the protein by means of a protonated Schiff base linkage to the ϵ-amino group of Lys^{296} located in the seventh transmembrane segment of the protein (Hargrave & McDowell, 1992; Khorana, 1992). The protonated nitrogen of the Schiff base is stabilized through an electrostatic interaction with a carboxylate residue in the third transmembrane segment, Glu^{113}, the Schiff base counterion (Zhukovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990). Upon absorption of light the chromophore isomerizes to the all-trans form, and the protein undergoes a series of thermally controlled conformational changes that result ultimately in an intermediate known as metarhodopsin II (meta II) which is responsible for activation of the G protein transducin (Matthews et al., 1963; Wald, 1968; Emeis et al., 1982). Inactivation of meta II is initiated by rhodopsin kinase, a member of the GRK family of Ser/Thr kinases (Palczewski & Benovic, 1991; Inglese et al., 1993) which is specific for light-activated rhodopsin (cf., Kuhn, 1984). Rhodopsin kinase phosphorylates the carboxyl-terminal tail of the activated rhodopsin allowing arrestin, another protein of the visual cascade, to bind and bring about the immediate inactivation of rhodopsin (Wilden et al., 1986; Bennett & Sitaramayya, 1988).

Mutation of several different amino acid residues in rhodopsin results in constitutive activation of the protein (Robinson et al., 1992; Cohen et al., 1993; Dryja et al., 1993; Rao et al., 1994). That is, these mutants are able to activate transducin in the absence of the chromophore and in the absence of light. Four of these amino acids are of particular interest because they activate the protein by a common mechanism of action; they disrupt a critical salt bridge between Lys^{296} and Glu^{113}. Two of the mutations disrupt the salt bridge by a direct mechanism, that is, by mutation of Lys^{296} or Glu^{113} (Robinson et al.. 1992). The other two mutations do so by an indirect mechanism. In these cases (Gly^{90} and Ala^{292}), a neutral amino acid residue located next to Lys^{296} in the wild-type protein is changed to a carboxylate side chain in the mutant. The newly introduced carboxylates compete with Glu^{113} for the positively charged Lys^{296} nitrogen, the critical salt bridge between 296 and 113 is disrupted, and the protein becomes active (Dryja et al., 1993; Rao et al., 1994).

These mutations are of interest not only for the insight they give us into activation and inactivation of rhodopsin but also because they have been found as the causative mutations in two different diseases of the retina. Mutations of Lys^{296} to Glu (Keen et al., 1991) or to Met (Sullivan et al., 1993) are known to cause autosomal dominant retinitis pigmentosa, whereas mutations of Gly^{90} to Asp (Sieving et al., 1995) and Ala^{292} to Glu (Dryja et al., 1993) cause stationary night blindness. It is very likely that the constitutively activated state of these receptors is responsible for the disease. In the case of stationary night blindness, activation of the receptors has been postulated to give rise to a signal causing the photoreceptor cells to desensitize in a process akin to normal light adaptation (Dryja et al., 1993; Rao et al., 1994). In the case of the retinitis pigmentosa mutations, a link connecting activated receptor to cell death has not yet been established although it seems reasonable to suggest that such a link does exist.

Recent studies on the phosphorylation of the K296G and K296E mutants have suggested that, although these mutant opsins constitutively activate transducin, they are not constitutively phosphorylated by rhodopsin kinase (Robinson et

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al., 1994). This conclusion has two important implications. First, is that the conformation of the constitutively active opsin mutants must be different from the conformation of the light-activated meta *II* state of wild-type rhodopsin which activates transducin and is phosphorylated by rhodopsin kinase (Robinson et al., 1994). Second, the K296E mutant found in certain patients with retinitis pigmentosa must continuously activate the phototransduction cascade in these individuals since K296E would not be inactivated by rhodopsin kinase and arrestin (Robinson et al., 1994).

The report that these mutants were not phosphorylated by rhodopsin kinase was highly surprising in part because this is contrary to the findings with constitutively active mutations in the β_2-adrenergic receptor where the mutants not only activate the G protein Gs, but are also constitutively phosphorylated by β-adrenergic receptor kinase (Pei et al., 1994). Given the importance of this issue to the mechanism of rhodopsin action and the implication for the molecular defect in retinitis pigmentosa caused by the constitutively active mutants, we have reexamined the question of whether or not the opsin mutants are constitutively phosphorylated by rhodopsin kinase. We show here that, in our hands, the constitutively active mutants K296G, K296E, K296M, E113Q, A292E, and G90D are all constitutively phosphorylated by rhodopsin kinase. Furthermore, the level of phosphorylation correlates with the relative activity of each mutant toward transducin. Finally, we also show that the phosphorylated mutants bind arrestin in a reaction that inhibits the subsequent interaction with transducin.

EXPERIMENTAL PROCEDURES

Materials. 11-*cis*-Retinal was the generous gift of Peter Sorter and Hoffman-La Roche (Nutley, NJ). The plasmid pCMV5-RK used to express rhodopsin kinase in COS cells was generously provided by James Inglese and Robert Lefkowitz (Duke University). Bovine retinae were obtained from J. A. Lawson Co. (Lincoln, NE). DE-52, BTP [1,3-bis[tris(hydroxymethyl)methylamino]propane], CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate], and Sepharose 4B were from Sigma (St. Louis, MO). Heparin Sepharose CL-6B was purchased from Pharmacia. Asolectin, from Associated Concentrates (Woodside, NY), was the gift of Dr. C. Miller (Brandeis University). Centricon 30 concentrators were from Amicon, Inc. (Beverly, MA). [^{35}S]-GTPγS (1156 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from NEN. Nonradiolabeled GTPγS (tetralithium salt) and ATP (disodium salt) were from Boehringer Mannheim and Pharmacia, respectively.

The anti-rhodopsin monoclonal antibody 1D4 (Molday and MacKenzie, 1983; MacKenzie et al., 1984) was purified from hybridoma culture medium (National Cell Culture Center, Minneapolis, MN) by $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography on DE-52 according to standard protocols as has been described (Oprian et al., 1987). The antibody was then coupled to the Sepharose 4B solid support by the method of Cuatrecasas (1970). Peptide I (AspGlu-AlaSerThrThrValSerLysThrGluThrSerGlnValAlaProAla), used for elution of opsin from the 1D4-Sepharose 4B matrix, was purchased from American Peptide Co., Inc. (Santa Clara, CA).

Urea-Stripped ROS. All procedures for preparation of urea-stripped ROS were performed in the dark with illumination from a 15 W incandescent bulb filtered through a Kodak safelight #2 filter. One hundred fifty milliliters of buffer A (20 mM Tris buffer, pH 7.4, 1 mM $CaCl_2$, and 2 mM DTT) containing 47% (w/v) sucrose and 0.15 mM PMSF was added to 100 thawed retinae and the suspension homogenized by passage through the luer-tip outlet of a 50 mL polypropylene syringe six times. The homogenate was then fractionated by centrifugation in a Beckman JA-20 rotor for 15 min at 19 000 rpm and 4 °C. The supernatant fraction containing the ROS, which floated to the top of the tube, was combined with 200 mL of buffer A, and the sample was centrifuged in a Beckman JA-14 rotor at 13 000 rpm for 20 min at 4 °C. The pellet was resuspended in 80 mL of buffer A and divided into six 15-mL aliquots. Each aliquot was then layered onto a discontinuous sucrose gradient composed of 8 mL of 30% (w/v) sucrose and 10 mL of 25% (w/v) sucrose in buffer A. The samples were then centrifuged in a Beckman SW 28.1 rotor at 27 000 rpm for 20 min at 4 °C. ROS were collected from the interface between sucrose solutions, diluted with 160 mL of 20 mM Tris buffer, pH 7.4, containing 1 mM $CaCl_2$, and collected by centrifugation in a JA-20 rotor at 19 000 rpm for 15 min at 4 °C. The ROS were washed two more times by resuspension and pelleting as just described, first in 100 mL of 10 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM $MgCl_2$, and 2 mM DTT, and then in 100 mL of buffer B (10 mM Tris buffer, pH 7.4, containing 0.1 mM EDTA and 2 mM DTT).

The final ROS pellet was then resuspended in 8 mL of buffer B containing 5 M urea. After a 5 min incubation on ice, the sample was diluted with 72 mL of buffer B and the stripped-ROS were isolated by centrifugation in a JA-20 rotor at 19 000 rpm for 20 min at 4 °C. The ROS pellet was washed twice with 100 mL of buffer B and resuspended in 10 mL of 10 mM Tris buffer, pH 7.4, containing 2 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF for storage at −70 °C.

Opsin Mutants. The wild-type opsin gene used in these studies was a synthetic gene encoding the amino acid sequence of native bovine rhodopsin (Ferretti et al., 1986). Mutations were constructed by cassette mutagenesis. With the exception of K296M, all mutant opsins used in this study have been described in previous publications: K296G (Zhukovsky et al., 1991; Robinson et al., 1992), K296E (Robinson et al., 1992), E113Q (Zhukovsky & Oprian, 1989; Robinson et al., 1992), A292E (Dryja et al., 1993), and G90D (Rao et al., 1994). These references should be consulted for details on construction of the mutant genes and the initial characterization of the proteins isolated from transfected COS cells. The gene for K296M was the generous gift of G. Cohen.

Expression of Wild-Type and Mutant Opsin Genes in COS Cells. The opsin genes were expressed transiently in transfected COS cells according to previously described protocols (Oprian et al., 1987; Oprian, 1993).

Purification of Wild-Type and Mutant Opsins. Transfected COS cells from 10 100-mm culture plates were harvested by scraping with a rubber policeman in 1 mL/plate 10 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl (PBS). The suspended cells were combined into a 15-mL conical, polypropylene culture tube and collected by centrifugation for 3 min in a clinical centrifuge. The supernatant fraction was discarded, and the cells were washed by

resuspending the pellet in 10 mL of 50 mM Tris buffer, pH 7.0, containing 140 mM NaCl and 1 mM dithiothreitol. The cells were collected again by centrifugation in the clinical centrifuge, the supernate was discarded, and the cell pellet was resuspended in 50 mM Tris buffer, pH 7.0, containing 140 mM NaCl, 1 mM dithiothreitol, 1% (w/v) CHAPS, and 10 mg/mL asolectin. This suspension was incubated with mixing at 4 °C for 20 min to solubilize cellular membranes and then fractionated by centrifugation for 3 min in a clinical centrifuge. The nuclear pellet was discarded and the supernate transferred to a fresh 15-mL conical polypropylene tube. 1D4-Sepharose 4B matrix (150 μL packed gel) was added and the suspension incubated at 4 °C with mixing for 35 min. The immunoaffinity beads were then collected by centrifugation for 1 min in a clinical centrifuge, the supernate was discarded, and the beads were transferred to a 1-mL polypropylene syringe barrel containing a glass wool plug in the bottom. The syringe was then placed in a 15-mL conical polypropylene tube, and the beads were washed by centrifugation 10 times with 1 mL of solubilization buffer. The protein was eluted from the solid support by resuspending the beads in 300 μL of solubilization buffer containing 50 μM peptide I followed by a 20 min incubation on ice. The eluate was collected by centrifugation, the elution procedure was repeated with a second 300 μL aliquot of peptide I, and the two fractions were combined for further use.

Reconstitution of Opsins into Lipid Vesicles. The eluate (approximately 600 μL) from the 1D4-Sepharose 4B matrix was applied to a 1 × 10 cm column of Sephadex G-50 that had been equilibrated with 10 mM Tris buffer, pH 7.4, containing 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol. The gel filtration column was developed under gravity flow with the same buffer, and fractions of 500 μL volume were collected and analyzed for light-scattering by recording the absorption spectrum from 650 to 500 nm. Turbid fractions from the void volume (usually two to three fractions) were pooled and the vesicles concentrated approximately 10-fold by centrifugation in a centricon-30 concentrator for 30 min at 7 000 rpm in a Beckman JA-20 rotor. The concentrated vesicles were collected (total volume approximately 150 μL) and stored at 4 °C until use.

Preparation of nPrSB. The Schiff base complex of 11-*cis*-retinal and *n*-propylamine (nPrSB) was prepared in ethanol solution as has been described previously (Zhukovsky et al., 1991).

Purification of Transducin. Transducin was purified from bovine retina according to the procedure of Wessling-Resnick and Johnson (1987) and then subjected to ion-exchange chromatography on DE-52 as described by Baehr et al. (1982). As a final step, the protein was dialyzed against 10 mM Tris buffer (pH 7.5) containing 50% (v/v) glycerol, 2 mM $MgCl_2$, and 1 mM dithiothreitol and then stored at −20 °C.

Preparation of Rhodopsin Kinase from Transfected COS Cells. Rhodopsin kinase was partially purified from COS cells transfected with the vector pCMV5-RK (Lorentz et al., 1991) by chromatography on heparin-Sepharose using a procedure developed by Palczewski (1993) for purification of the protein from bovine retina. The procedures for growth and transfection of COS cells were the same as those used for expression of the opsin genes (Oprian et al., 1987; Oprian, 1993) except that the growth medium contained 4 mM mevalonic acid lactone to ensure complete isoprenylation of the kinase (Inglese et al., 1992). Typically, three to five 100-mm culture plates of COS cells were transfected with 15 μg/plate of pCMV5-RK using DEAE-dextran and the cells harvested 48 h later. Cells were scraped from the plates with a rubber policeman in 1 mL PBS/plate and collected by centrifugation in a clinical centrifuge. The cells were lysed at 4 °C by resuspension in 8 mL of 10 mM BTP buffer, pH 7.5, containing 0.4% (v/v) Tween-80. This suspension was then centrifuged in a Beckman Ti 70.1 rotor for 45 min at 50 000 rpm and 4 °C. The supernatant fraction was loaded by gravity flow (approximately 20 mL/h) onto an heparin-Sepharose column (1 × 10 cm) that had been equilibrated with 10 mM BTP buffer, pH 7.5, containing 0.4% (v/v) Tween-80. The column was then washed successively with (1) the 10 mM BTP buffer, pH 7.5, containing 0.4% (v/v) Tween-80 until the eluant absorbance at 280 nm fell below 0.01, (2) 10 mM BTP buffer, pH 7.5, containing 125 mM KCl, 1 mM $MgCl_2$, and 0.4% (v/v) Tween-80, again until the eluant absorbance fell below 0.01, and (3) 6 mL of 10 mM BTP buffer, pH 7.5, containing 100 mM KCl, 1 mM $MgCl_2$, and 0.4% (v/v) Tween-80. After the final wash, 10 mM BTP buffer, pH 7.5, containing 100 mM KCl, 1 mM $MgCl_2$, 0.4% (v/v) Tween-80, and 0.2 mM ATP was applied to the column until a volume corresponding to the void volume of the column (approximately 3 mL) had eluted, and then the column flow was stopped. After a 30 min incubation period, 2 mL fractions were collected and assayed for rhodopsin kinase activity using urea-stripped ROS. Active fractions were then pooled (approximately 14 mL total volume), concentrated (Centricon-30) approximately 40-fold, and stored at 4 °C until use. Rhodopsin kinase was not used after 2 weeks of storage. When arrestin binding to phosphorylated rhodopsin and mutants was measured, the rhodopsin kinase used in the phosphorylation reaction was purified essentially as described above except that the Tween-80 was omitted from the protocol.

Purification of Rhodopsin Kinase from Bovine Retina. Rhodopsin kinase was purified from bovine retina exactly as has been described by Palczewski (1993). The specific activity of the purified protein was determined to be 165 nmol of phosphate min^{-1} (mg of rhodopsin kinase)$^{-1}$ by phosphorylation of urea-stripped bovine ROS with [γ^{32}P]-ATP (1000 cpm/pmol) as described by Palczewski (1993). Protein concentration was determined by the method of Lowry et al. (1951) as modified by Bensadoun and Weinstein (1976).

Purification of Arrestin from Bovine Retina. Arrestin was purified from bovine retina according to the protocol of Buczylko and Palczewski (1993). The concentration of purified arrestin was determined from the ultraviolet absorption maximum at 278 nm using an extinction coefficient of 0.638 for a 0.1% solution (Buczylko & Palczewski, 1993).

Assay for Activation of Transducin. Wild-type and mutant opsins were assayed for their ability to catalytically activate transducin by following the binding of [^{35}S]GTPγS as has been described previously (Zhukovsky et al., 1991; Cohen et al., 1992) except that the opsins were assayed in asolectin vesicles. Unless noted otherwise, reaction mixtures contained 2.5−5 nM opsin in 10 mM Tris buffer, pH 7.5 (or 10 mM MES buffer, pH 6.5, as indicated in the text), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 2.5 μM transducin, and 3 μM [^{35}S]GTPγS (3 Ci/mmol). The

reactions were initiated by addition of GTPγS. The temperature was 24−26 °C. For light-dependent reactions, the opsin-containing vesicles were incubated in a solution of 10 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, and 11-*cis*-retinal (or nPrSB in the case of K296G) at 180 μM final concentration for 1 h in the dark at room temperature before dilution (10-fold) into the reaction mixture.

pH-Rate Profile for K296M. The pH-rate profiles for activation of transducin by all constitutively active opsin mutants, as well as for light-activated rhodopsin, are bell-shaped with the lower apparent pK_a referred to as pK_{a1} and the higher pK_a referred to as pK_{a2}. To determine the values of pK_{a1} and pK_{a2} for the K296M mutant, a pH-rate profile was determined as previously described (Cohen et al., 1992, 1993), and the data were simulated with the equation [see Cohen et al. (1992, 1993)]

$$\text{obsd rate} = V_{max}[H/(H + K_{a2})][K_{a1}/(H + K_{a1})] + T \quad (1)$$

where H is the proton concentration and the expression $[H/(H + K_{a2})][K_{a1}/(H + K_{a1})]$ is the fraction of the protein that is both protonated at site(s) 2 and unprotonated at site(s) 1 at proton concentration H. It is important to emphasize here that the number of groups ionizing in either of these transitions (pK_{a1} or pK_{a2}) is unknown. Only the net number of protons involved in the transition may be determined from the data. The term T (eq 1) has been added to account for the pH-dependent activation of transducin that occurs in the absence of opsin (Cohen et al., 1992).

Assay for Phosphorylation by Rhodopsin Kinase. Phosphorylation of wild-type and mutant opsins was measured using the COS cell expressed proteins after reconstitution into asolectin vesicles. The reaction mixture contained in a total volume of 30 μL of 75 mM Tris buffer, pH 7.5 (or MES buffer, pH 6.5), 10 mM $MgCl_2$, 5 mM DTT, 100 μM [γ-^{32}P]ATP (2000 cpm/pmol), a 6 μL aliquot of the partially purified and concentrated rhodopsin kinase, and 15 pmol opsin in asolectin vesicles (the concentration of opsin was estimated from Western blots). The reactions were initiated by the addition of rhodopsin kinase. For light-dependent phosphorylations, the reactions were initiated by exposure to white light which was continuous throughout the incubation period. The reactions were allowed to proceed for the indicated amount of time and then stopped by the addition of 8 μL of 5× SDS load buffer (300 mM Tris buffer, pH 6.8, 10% SDS, 30% sucrose, and 0.026% bromophenol blue) and 2 μL of β-mercaptoethanol. The proteins were then separated by SDS−PAGE (entire sample was loaded onto 10% gels: 10% acrylamide, 0.27% bisacrylamide), the gels dried on filter paper using a Bio-Rad Model 583 gel dryer (1 h at 60 °C), and the phosphorylated proteins detected by autoradiography on Kodak X-OMAT AR film using an intensifying screen at −70 °C.

Assay for Arrestin Activity. Arrestin activity was assayed by measuring its ability to inhibit the activation of transducin by phosphorylated rhodopsin. Phosphorylation of the opsin and rhodopsin samples was carried out essentially as described above with the following exceptions: (1) the opsin concentration was 100 nM in the reaction mixture; (2) 10 instead of 6 μL of partially purified COS cell rhodopsin kinase was used in the assay; and (3) the ATP (100 μM) was not radiolabeled. Phosphorylation was allowed to proceed for 1 h at room temperature. Control samples were treated identically except that no rhodopsin kinase was added to the reaction mixture. When present, 50 μM arrestin was added to the samples during the last 20 min of incubation. At the end of 1 h the samples were diluted 10-fold into the transducin reaction mixture and assayed for ability to activate transducin as described above.

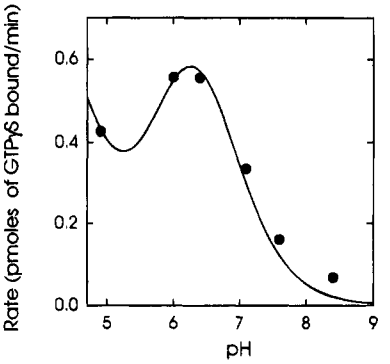

FIGURE 1: pH-rate profile for transducin activation by K296M opsin in the absence of chromophore. Closed circles are rates for transducin activation determined from the initial linear portion of each reaction time course. The solid line was generated from eq 1 under Experimental Procedures. pK_{a1} and pK_{a2} values are 5.9 and 6.7, respectively.

RESULTS

pH-Rate Profile for K296M. The mutant K296M is constitutively active as would be expected from a neutral amino acid side chain at position 296 (Robinson et al., 1992). The pH-rate profile for activation of transducin by K296M is shown in Figure 1. The profile is bell-shaped and was fit well by eq 1 from Experimental Procedures where pK_{a1} = 5.9 and pK_{a2} = 6.7. The value of pK_{a2} has previously been shown to be a highly sensitive function of the size and charge of amino acid side chain at position 296 (Cohen et al., 1993). A value of 6.7 is consistent with the pK_{a2} expected for a mutant opsin with Met at this position (Cohen et al., 1993), a neutral amino acid residue with a volume of 163 Å^3 (Creighton, 1984).

Phosphorylation of Constitutively Active Mutants of Rhodopsin. It is well known that rhodopsin becomes a substrate for phosphorylation by rhodopsin kinase only after exposure of the protein to light. This is illustrated in Figure 2A where bovine rhodopsin in urea-stripped ROS membranes shows no phosphorylation when incubated with rhodopsin kinase in the dark (lane 1) but rapidly becomes phosphorylated upon illumination (lane 2). Similarly, phosphorylation of rhodopsin from transfected COS cells is completely dependent upon exposure to light (Figure 2B). Neither purified opsin (lane 1) nor rhodopsin (lane 2) are phosphorylated when incubated with rhodopsin kinase in the dark, but the COS cell rhodopsin becomes rapidly phosphorylated upon illumination (lane 3). In stark contrast, the constitutively active mutant K296G is constitutively phosphorylated when incubated with rhodopsin kinase in the absence of retinal chromophore (Figure 2D, lane 1). There is no phosphorylation of this mutant if the protein is first reconstituted with the *n*-propyl Schiff base of 11-*cis*-retinal (nPrSB) and then incubated with rhodopsin kinase in the dark (lane 2). However, phosphorylation of the mutant rhodopsin is restored upon exposure to light (lane 3). The K296G mutant is constitutively phosphorylated by

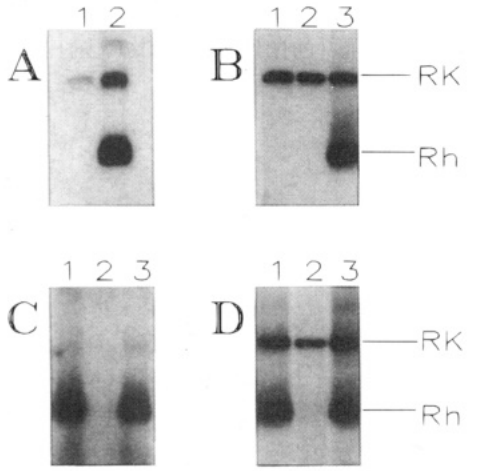

FIGURE 2: Phosphorylation of wild-type rhodopsin and mutant K296G by rhodopsin kinase. The figure shows autoradiograms of rhodopsin and the K296G mutant phosphorylated with rhodopsin kinase *in vitro* using [γ-^{32}P]ATP. (A) Phosphorylation of wild-type rhodopsin from urea-stripped ROS membranes. Lane 1, phosphorylation in the dark; lane 2, phosphorylation after exposure of the sample to light. (B) Phosphorylation of wild-type rhodopsin from transiently expressed COS cells. Lane 1, phosphorylation of the wild-type COS cell opsin, that is, the isolated protein before addition of the 11-*cis*-retinal chromophore; lane 2, phosphorylation of the wild-type COS cell rhodopsin (i.e., COS cell opsin after addition of 11-*cis*-retinal) in the dark; lane 3, phosphorylation of the wild-type COS cell rhodopsin after exposure to light. (C and D) Phosphorylation of the K296G mutant opsin. Lane 1, phosphorylation in the absence of added chromophore; lane 2, phosphorylation in the dark after addition of nPrSB; lane 3, phosphorylation of the K296G rhodopsin after exposure to light (i.e., the sample from lane 2 after exposure to light). Rhodopsin kinase was isolated either from transfected COS cells (A, B, and D) or from bovine retina (C). All reactions shown in this figure were conducted at pH 7.5 for 30 min. Rh indicates location of phosphorylated rhodopsin; RK indicates location of autophosphorylated rhodopsin kinase.

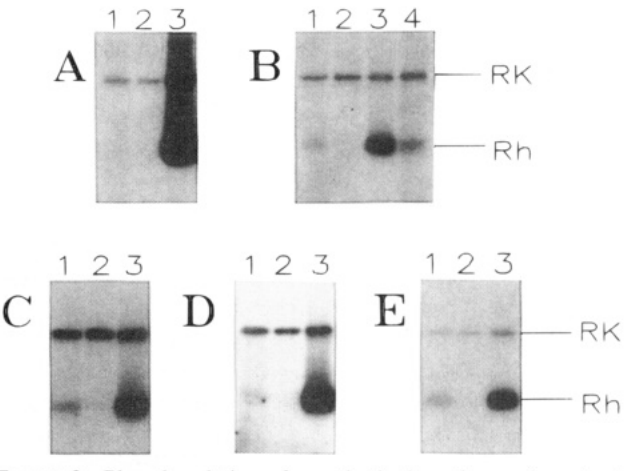

FIGURE 3: Phosphorylation of constitutively active opsin mutants by rhodopsin kinase. The figure shows autoradiograms of wild-type and mutants phosphorylated with rhodopsin kinase *in vitro* using [γ-^{32}P]ATP. A, wild-type; B, K296M (lanes 1 and 2) and K296E (lanes 3 and 4); C, E113Q; D, G90D; and E, A292E. In A and C−E: lane 1, phosphorylation of the opsin forms in the absence of added 11-*cis*-retinal; lane 2, phosphorylation in the dark after addition of 11-*cis*-retinal; and lane 3, phosphorylation of sample from lane 2 after exposure to light. In B, the K296M (lanes 1 and 2) and K296E (lanes 3 and 4) mutants cannot bind 11-*cis*-retinal so that only the opsin forms are shown. In each case the phosphorylation reaction is shown at pH 6.5 (lanes 1 and 3) and pH 7.5 (lanes 2 and 4). All phosphorylation reactions shown in this figure were performed with COS cell rhodopsin mutants and rhodopsin kinase. Rh indicates location of phosphorylated rhodopsin; RK indicates location of autophosphorylated rhodopsin kinase. The pH was as indicated above for B and 6.5 for A and C−E. The phosphorylation reactions were allowed to proceed for 40 min for parts A and B and for 30 min for parts C-E.

rhodopsin kinase irrespective of whether the kinase was partially purified from transfected COS cells (Figure 2D) or highly purified from bovine retina (Figure 2C).

These data are reminiscent of the behavior of the K296G mutant in assays for the activation of transducin (Robinson et al., 1992). K296G activates transducin constitutively in the absence of the chromophore, this activity is turned off in the dark by incubation with nPrSB, and the activity returns upon exposure of the protein to light.

The naturally occurring mutants K296E (Keen et al., 1991) and K296M (Sullivan et al., 1993) found in certain patients with retinitis pigmentosa are also constitutively phosphorylated by rhodopsin kinase, as is shown in Figure 3B. The activity of these mutants was less than that of K296G, both as a consequence of lower expression levels and a decreased intrinsic activity of the mutant (in the case of K296M). For this reason the pH of the reaction mixture was changed from 7.5 (as in Figure 2) to 6.5. Constitutive activation of transducin is dramatically increased at this pH, as shown for K296M in Figure 1, and the phosphorylation activity is also increased as is shown in Figure 3B. As a control, wild-type opsin from COS cells was also assayed at pH 6.5 (Figure 3A). As is evident from the deliberately overexposed autoradiogram, the wild-type opsin was not detectably phosphorylated under these conditions (Figure 3A, lane 1).

The mutant opsins G90D, E113Q, and A292E are all phosphorylated constitutively by rhodopsin kinase (Figure 3D, C, and E, respectively). Reconstitution with 11-*cis*-retinal inhibits phosphorylation in the dark, and the activity is restored upon exposure to light.

It is noteworthy that there is a rough correlation of the ability of these mutants to constitutively activate transducin (Robinson et al., 1992; Dryja et al., 1993; Rao et al., 1994) with their ability to serve as substrates for rhodopsin kinase. If we narrow this analysis to those mutants that can be reconstituted with a retinal chromophore (to control for different expression levels), the ability to activate transducin decreases in the order K296G > E113Q > A292E > G90D. As can be seen in Figures 2 and 3, constitutive phosphorylation of the mutants by rhodopsin kinase also decreases in the order K296G > E113Q > A292E > G90D.

Arrestin Inhibition of Phosphorylated Opsin Mutants. As is shown in Figure 4, constitutive activation of transducin by all of the mutants used in this study (K296G, K296E, K296M, E113Q, G90D, and A292E), as well as the light-dependent activation of transducin by wild-type rhodopsin isolated from COS cells, was inhibited by the combined action of rhodopsin kinase and arrestin. Phosphorylation of the mutants by rhodopsin kinase alone was insufficient for inactivation of the proteins, as also was incubation with arrestin in the absence of rhodopsin kinase.

DISCUSSION

The major conclusion to be drawn from this study is that mutations of rhodopsin that result in constitutive activation of transducin also result in constitutive phosphorylation by rhodopsin kinase. This was first demonstrated for the mutant K296G. K296G is constitutively phosphorylated in the

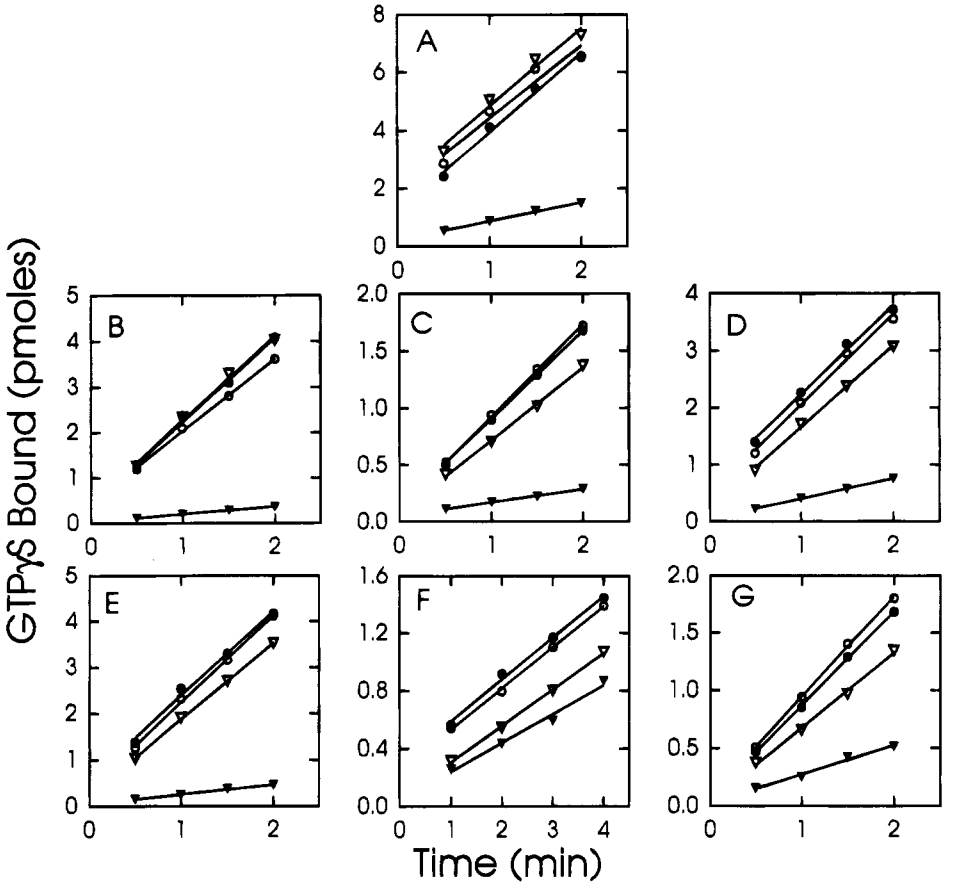

FIGURE 4: Effect of rhodopsin kinase and arrestin on the constitutive activity of opsin mutants. A, wild-type rhodopsin; B, K296G; C, K296E; D, K296M; E, E113Q; F, G90D; and G, A292E. Open circles, activation of transducin in the absence of rhodopsin kinase and arrestin; closed circles, activation in the presence of arrestin; open triangles, activation after incubation with rhodopsin kinase; closed triangles, activation after incubation with rhodopsin kinase and in the presence of arrestin. The assay for activation of transducin, phosphorylation of rhodopsin by rhodopsin kinase, and quenching of the reaction by arrestin was as described under Experimental Procedures. The reaction with wild-type rhodopsin (A) was performed with rhodopsin after reconstitution of the sample with 11-*cis*-retinal and exposure to light. All others (B−G) were performed in the absence of added chromophore where only constitutive activity is monitored. The pH of the different samples differed as follows: A−C, pH 7.5; D−G, pH 6.5. The inhibition of G90D by arrestin was weaker and more variable than that observed with the other mutants. This is presumably a consequence of the low specific activity of G90D.

absence of any chromophore. Phosphorylation is inhibited if K296G is first reconstituted with the *n*-propyl Schiff base of 11-*cis*-retinal and incubated with rhodopsin kinase in the dark, but phosphorylation of the protein is restored upon exposure to light. This behavior is essentially identical to that previously reported for the activation of transducin by this mutant (Robinson et al., 1992). It is also reminiscent of activating mutations in the α_2- and β_2-adrenergic receptors where point mutations in the intracellular loop connecting transmembrane segments 5 and 6 result in constitutive activation of the G proteins (Samama et al., 1993; Ren et al., 1993) and constitutive phosphorylation of the receptor by β-adrenergic receptor kinase (Ren et al., 1993; Pei et al., 1994). As a consequence, the β_2-receptor is desensitized and down-regulated in Chinese hamster ovary cell lines carrying the mutation (Pei et al., 1994).

Additionally, we have shown here that all of the constitutively active mutants tested in this study also are constitutively phosphorylated by rhodopsin kinase. These include the counterion mutant, E113Q, the two retinitis pigmentosa mutants, K296E and K296M, and the two night blindness mutants, G90D and A292E. Furthermore, we showed that arrestin binds to the phosphorylated proteins in a reaction that quenches activation of transducin.

There are two conclusions to be drawn from these results. First is that the conformation of opsin in the constitutively active mutants may be similar to that of metarhodopsin II in the photoreaction. This is not proven by these results. However, we note that metarhodopsin II not only activates transducin but is also phosphorylated by rhodopsin kinase. Similarly, the constitutively active mutants not only activate transducin but are also phosphorylated by rhodopsin kinase. Additionally, there is a rough correlation of activity toward transducin with activity toward rhodopsin kinase in these mutants. The order of activity in both reactions is K296G > E113Q > A292E > G90D. Therefore it seems reasonable to conclude that transducin and rhodopsin kinase are recognizing the same conformational change brought about by the mutations.

The second conclusion to be drawn from the results is that the constitutively active retinitis pigmentosa mutants K296E and K296M may not activate transducin in photoreceptor cells from these patients. Instead, the proteins may be perpetually phosphorylated and bound in inactive complexes with arrestin. Indeed, this is exactly what has been observed in a recent study by Li et al. (1995) of the K296E mutant in a transgenic mouse model of the disease. They show that the K296E mutant does not activate transducin

constitutively *in vivo*. The reason apparently is that the mutant protein is phosphorylated and bound with arrestin. Upon extraction of the ROS, removal of peripheral proteins with urea, and treatment with phosphatase 2A, the K296E mutant displays the same constitutive activity that is observed under *in vitro* conditions.[1]

What then is the molecular defect that causes retinitis pigmentosa in patients with K296E and K296M? One possibility is that the binding of arrestin to phosphorylated opsin (or the phosphorylation per se) competitively disrupts essential interactions with other proteins in the rod cell. This is an intriguing possibility because it suggests that the underlying defect in these patients may be similar to that of certain patients with class I mutations (Sung et al., 1991) in rhodopsin. For example (Sung et al., 1994), the Q344ter mutant, which is truncated at position 344 in the carboxy-terminal tail, reconstitutes with 11-*cis*-retinal normally when expressed in HEK 293S cells, activates transducin normally in a light-dependent manner, and is phosphorylated by rhodopsin kinase in a reaction that is indistinguishable from that of the wild-type. However, in transgenic mice the protein appears to have a defect in localization to the photoreceptor outer segment, and it is thought that this results from disrupted interactions with rod cell proteins required for transport or retention of opsin in the photoreceptor outer segment (Sung et al., 1994). Immunohistochemical staining of rod photoreceptor cells in the K296E transgenic mice (Li et al, 1995) does not support a model for disrupted transport of this mutant to the rod outer segment, as has been shown for the Q344ter mutant (Sung et al., 1994) and other rhodopsin mutants causing retinal degeneration in *Drosophila* (Colley et al., 1995; Kurada & O'Tousa, 1995), and further work will be required to fully evaluate this model and identify the underlying defect responsible for retinal degeneration in photoreceptor cells harboring the mutants K296E and K296M.

We note that the two night blind mutants, A292E (Dryja et al., 1993) and G90D (Rao et al., 1994; Sieving et al., 1995), behave completely differently in this regard than the two retinitis pigmentosa mutants. A292E and G90D do not have mutations in the active site Lys^{296}. If activated by thermal dissociation of 11-*cis*-retinal from the protein, they will be phosphorylated and the phosphorylated protein will bind arrestin in reactions that compete with activation of transducin, as is the case for wild-type (Wilden et al., 1986). However, once the phosphorylated protein is bound in an inactive complex with arrestin, 11-*cis*-retinal can bind to the mutant to release arrestin and allow dephosphorylation with phosphatase 2A (Palczewski et al., 1989; Hoffman et al., 1992; Ohguro et al., 1995). In this model, the loss of function in rod photoreceptor cells results from adaptation to an activating signal arising from the spontaneous dissociation of retinal from the mutant opsins (Rao et al., 1994). In contrast, the retinitis pigmentosa mutants K296E and K296M have mutations in the active site Lys. Therefore, once phosphorylated and bound with arrestin, retinal cannot reverse these reactions, and the inactive complexes accumulate in the cell.

Finally, we note that the conclusion presented here that K296G and K296E are constitutively phosphorylated by rhodopsin kinase is different from that reported by Robinson et al. (1994). Despite significant effort from both groups, we currently have no explanation for the differences in our results.

[1] In the experiments of Li et al. (1995), K296E had to be dephosphorylated before constitutive activation of transducin was observed. This is somewhat different from what we have reported here. The difference may reflect a greater extent of phosphorylation (Miller et al., 1986) under *in vivo* conditions or perhaps the involvement of a kinase other than rhodopsin kinase (Newton & Williams, 1993).

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