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Constitutive activation of phototransduction by K296E opsin is not a cause of photoreceptor degeneration

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ABSTRACT **The missense mutation Lys-296 → Glu (K296E) in the rhodopsin gene produces an opsin with no chromophore binding site and therefore is not activated by light. Nevertheless, the mutant opsin constitutively activates transducin *in vitro* and causes photoreceptor degeneration *in vivo*, possibly by continuously activating the phototransduction cascade, analogous to constant exposure to environmental light. We studied the K296E mutation in eight lines of transgenic mice. Each line developed photoreceptor degeneration with the rate of degeneration increasing monotonically as the ratio of mutant:wild-type opsin mRNA increased. At no time in the course of degeneration was there endogenous light adaptation in the retina as measured by the electroretinogram. The mutant opsin was found to be invariably phosphorylated and stably bound to arrestin. Light-independent activation of transducin was demonstrated only after the removal of arrestin and dephosphorylation of K296E opsin. Thus, K296E opsin *in vivo* does not activate the phototransduction cascade because it is shut off by photoreceptor inactivation mechanisms. Our data show that the K296E mutation does not cause photoreceptor degeneration by continuous activation of phototransduction.**

Over 60 different mutations in the rhodopsin gene have been found to cause autosomal dominant retinitis pigmentosa (RP), a progressive degeneration of the neural retina that typically leads to blindness in middle age. The mechanisms by which any of these mutant alleles leads to degeneration of the rod and cone photoreceptors remain obscure. One testable hypothesis (1) is that the mutant opsins continuously activate transducin, resulting in a pathogenic overstimulation of photoreceptors. Support for this hypothesis comes from several observations. Constant exposure to light damages photoreceptor cells (2, 3). Many rhodopsin mutants regenerate poorly with the chromophore, 11-*cis*-retinal. Opsin, the apoprotein without the chromophore, was reported to activate phototransduction without light [albeit at six orders of magnitude lower efficiency compared to photo-activated metarhodopsin II (4)]. Thus defective regeneration might lead to a pool of weakly active opsin molecules in the photoreceptors. In addition, a rhodopsin mutant, K296E, that causes RP (5, 24) has been found to activate transducin constitutively *in vitro* (6). To gain insight into the consequences of a constitutively active mutant *in vivo*, we generated transgenic mice bearing the K296E (Lys-296 → Glu) mutation. We examined the characteristics of retinal degeneration in these mice and analyzed the mutant opsin from the transgenic mouse retinas.

MATERIALS AND METHODS

Construction of Transgene and Generation of Transgenic Mice. The K296E transgene was generated by site-directed mutagenesis on a genomic DNA fragment encompassing the normal human opsin gene, which was 16 kb in length and contained 4.8 kb of upstream and 6.2 kb of downstream sequences (7). Codon 296 was altered from AAG(Lys) to GAG(Glu). Microinjection of transgene DNA into fertilized eggs (Fvb/nJ) was carried out as described (8). Founders were mated to C57BL/6 mice, as were all subsequent generations. Lines with the same letter designation followed by different numbers were derived from a single founder. The transgene mRNA was sequenced following reverse transcription and PCR amplification and found to contain the engineered mutation. Mice were maintained under 12-hr light/12-hr dark cycles. A previously characterized transgenic mouse, NHR (normal human rhodopsin, E line; ref. 7), that does not develop photoreceptor degeneration was included in this study as a control to distinguish between potential effects of human opsin in mouse retina and the specific effects of K296E mutation. C57BL/6 mice were used as wild-type (wt) controls. Determination of transgene/endogenous opsin mRNA ratio was carried out essentially as described (7). We note here that the sequence for primer 1126 as given in ref. 7 is incorrect and should be (5′-CCGAAGCGGAAGTTGCTCAT-3′).

Electroretinogram (ERG) Testing. Mice were dark-adapted for at least 12 hr before testing. Testing was conducted as described (7), except that electrodes were placed in direct contact with the cornea instead of in the vitreous. With our setup, normal b-wave amplitudes in adult wt mice fall in the range between 500 and 1200 μV and implicit times range between 45 and 75 msec.

Immunocytochemistry. Frozen retinal sections were stained with primary antibodies and Cy3-conjugated secondary antibodies as described (7, 9). The monoclonal antibody rho 3A6 (gift of Robert Molday, University of British Columbia, Vancouver) is specific for human opsin (10). A11-82P monoclonal antibody (gift of Paul Hargrave, University of Florida, Gainesville) is specific for phosphorylated opsin (11). The arrestin antibody (gift of Igal Gery, National Institutes of Health, Bethesda, MD) is a rabbit antiserum against HPLC-purified bovine arrestin and cross-reacts with rat arrestin (12). Where applicable, mice were dark-adapted for 12 hr or light-adapted under bright white light for 30 min before experimentation.

Rod Outer Segment (ROS) Preparation and Analysis. Dark-adapted retinas were dissected under an infrared microscope. About 10 retinas were pooled for each preparation. ROS were isolated essentially as described (13). Unless noted, all subsequent manipulations were carried out in the dark or under dim red light. Rhodopsin was quantified by differential spectrophotometry. Briefly, ROS were solubilized in 2% Ammonyx LO/100 mM sodium phosphate buffer, pH 7.0, and scanned between 250 and 650 nm on a model 940 Uvicon spectrophotometer before and after photobleaching. The difference in absorption at 500 nm was used to calculate rhodopsin con-

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Abbreviations: wt, wild-type; ERG, electroretinogram; RP, retinitis pigmentosa; ROS, rod outer segment(s).

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Table 1. Correlation between the level of transgene expression and severity of retinal degeneration

Line	Transgene:endogenous opsin mRNA level	Retinal degeneration phenotype
A	0.25:1	Slow, ≈50% b-wave amplitude remaining at 7–8 months
F-2	1:1	Intermediate, see Fig. 1
G-2	3:1	Intermediate to fast, ≈10% b-wave amplitude remaining at 35 days
B-2	3:1	Similar to G-2
F-1	3:1	Similar to G-2
E	10:1	Very fast, nearly flat ERG at 30 days
C	14:1	Similar to E
I	15:1	Similar to E
NHR	1:1	No degeneration

centration assuming an extinction coefficient of 42,000 $M^{-1}\cdot cm^{-1}$. The yield of purified ROS was 0.1–0.2 nmol of rhodopsin per eye, or about 20–40% of the rhodopsin content measured in total eye extracts. Total ROS proteins were quantitated by the Lowry assay. Comparison of the two values indicated about 70% of the total protein was rhodopsin. *In vitro* phosphorylated opsin was prepared as described (14). ROS were either used without further treatment or washed with a hypotonic buffer [5 mM Tris, pH 7.5/0.5 mM $MgCl_2$/0.5 mM dithiothreitol (DTT)/1 mM phenylmethylsulfonyl fluoride], and the washed ROS membranes were recovered by centrifugation at 100,000 × *g* in a Beckman TLS-55 rotor for 15 min. To prepare urea-stripped ROS, hypotonically washed ROS membranes were resuspended in 4.5 M urea in 5 mM Tris, pH 7.5/0.5 mM $MgCl_2$/0.5 mM DTT, incubated on ice for 10 min, and pelleted again as above. Dephosphorylation of rhodopsin was carried out by incubating 5 pmol of urea-stripped rhodopsin with 0.04 unit of protein phosphatase $2A_1$ (Calbiochem; ref. 15) and 0.5 μg of histone H1 in 10 μl of buffer (10 mM Tris, pH 7.5/100 mM NaCl/5 mM $MgCl_2$/1 mM DTT) at room temperature for 2 hr. Assay for transducin activation using washed ROS membranes was carried out as described (6, 16). Purified bovine transducin was a gift from Thomas Sakmar (Rockefeller University, New York).

RESULTS

Characterization of K296E Mice. Eight lines of K296E mice were examined. Light microscopy of retinal sections showed a wide range of photoreceptor degeneration among the lines, from shortened rod outer segments to complete absence of the outer nuclear layer. The severity of retinal degeneration correlated with the ratio of transgene mRNA to endogenous mouse opsin mRNA characteristic of each line, which varied from 0.25:1 to 15:1 (Table 1). The ERG amplitudes also correlated with the degree of photoreceptor cell loss and the correlation was comparable to those found previously in transgenic mice carrying other mutant opsin alleles (7, 17). In the A line with the lowest transgene expression, the ERG and outer nuclear layer appeared normal initially. After 7 months of age, a loss of photoreceptor cells was evident, and there was an accompanying reduction in ERG amplitudes. In the lines with higher levels of transgene expression, the ERGs showed prolonged a- and b-wave implicit times and reduced to nondetectable amplitudes at the earliest ages tested (15–35 days). Fig. 1 shows representative light microscopic findings and ERGs from the F-2 line, which has approximately equal amounts of transgenic and endogenous opsin mRNA and an intermediate phenotype.

Overexpression of normal human opsin in transgenic mice, as measured by mRNA ratios, causes retinal degeneration (7). The threshold ratio of transgene:endogenous opsin mRNA above which degeneration will occur is ≈2-3:1 (18). For the experiments described below we chose two lines (A and F-2) with ratios ≤1:1, since their disease was attributable to the pathogenic effect of the K296E mutation rather than a deleterious effect of overexpression.

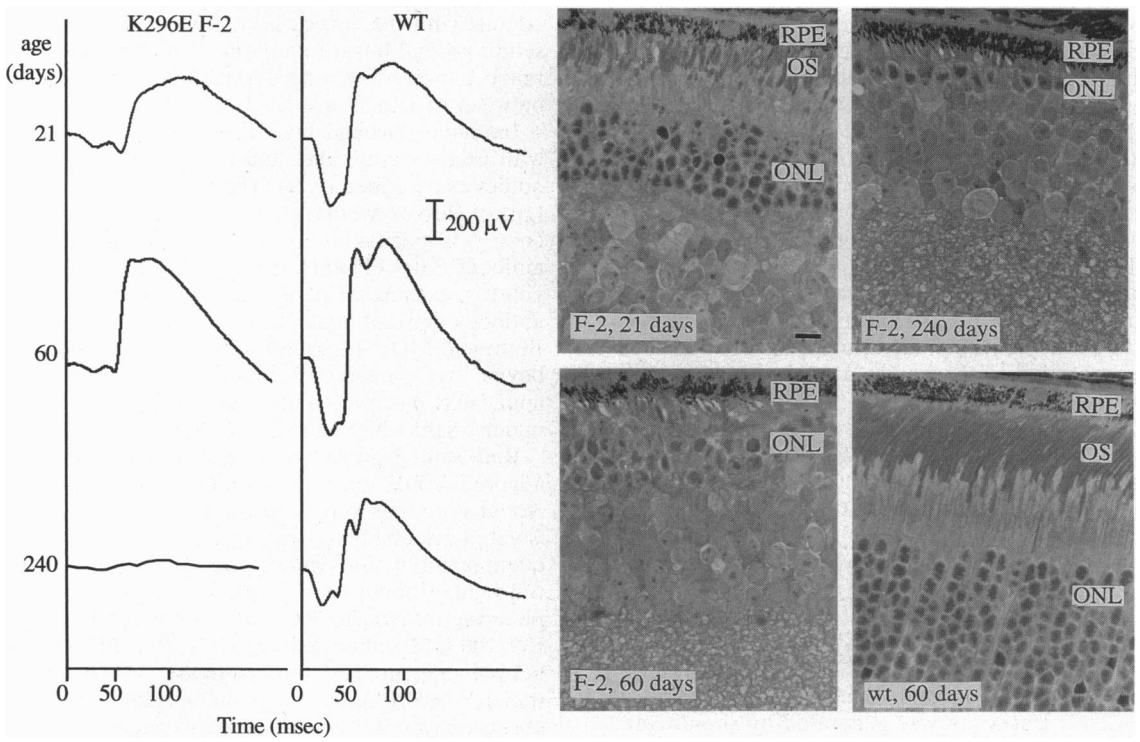

FIG. 1. (*Left*) Computer-averaged full-field ERGs in response to 0.1-Hz flashes of white light, recorded from K296E F-2 mice and age-matched wt controls. Each tracing is the computer-averaged composite of four separate responses. At 21 days, K296E F-2 mice ($n = 5$) had a b-wave amplitude of 371 ± 124 μV and a b-wave implicit time of 85 ± 16 msec. (*Right*) Light micrographs of retinas from transgenic mice and a wt control. OS, photoreceptor outer segment; ONL, outer nuclear layer; RPE, retinal pigment epithelium. (Bar = 20 μm.)

Localization of K296E Opsin. The expression of K296E opsin in the photoreceptor cells of transgenic mice was confirmed by immunocytochemical staining of frozen retinal sections with the human opsin-specific antibody rho 3A6 (data not shown). The mutant opsin was distributed predominantly in the ROS. In retinas with advanced degeneration, there was accumulation of opsin in the outer nuclear layer, which has been noted in other mouse models of RP (19).

K296E Opsin Is Phosphorylated Independent of Light. To explain the apparent lack of light-independent activity in the K296E retina as suggested by ERG, we hypothesized that the K296E opsin may be shut off *in vivo* by phosphorylation and subsequent binding to arrestin. The phosphorylation status of the mutant opsin was probed *in situ* by immunofluorescence with the A11-82P antibody, which is specific for phosphorylated opsin (Fig. 2). As expected, this antibody reacted with light-adapted wt, NHR, and K296E retinas. After dark-adaptation, however, only K296E retinas reacted, indicating that phosphorylated opsin was present. To gain additional evidence that the reacting antigen was opsin rather than a cross-reacting antigen, we probed K296E ROS proteins with antibody A11-82P on immunoblots. A band comigrating with *in vitro* phosphorylated opsin was detected (Fig. 3). The staining in the dark-adapted transgenic retinas, especially in A line, was weaker than that in the light-adapted retinas (Figs. 2 and 3), primarily due to a lower abundance of mutant opsin relative to wt opsin.

K296E Opsin Is Bound to Arrestin. In wt retinas, arrestin is present in the inner and outer segments of dark-adapted photoreceptors and shifts to outer segments upon photobleaching as a result of binding to phosphorylated rhodopsin (14, 20). To determine whether phosphorylated K296E opsin was able to bind arrestin, we first evaluated immunocytochemical staining with an anti-arrestin antibody. Although a stronger staining was noted in the outer segments of dark-adapted K296E (A line) photoreceptors compared to wt and NHR controls (not shown), it was difficult to quantify. We then compared the amount of arrestin present in hypotonically washed ROS membranes from dark-adapted transgenic and control retinas by immunoblotting. Fig. 4 shows that arrestin present in the wt and NHR ROS was apparently unbound, as it was lost after the hypotonic wash. In contrast, much of the arrestin in K296E ROS

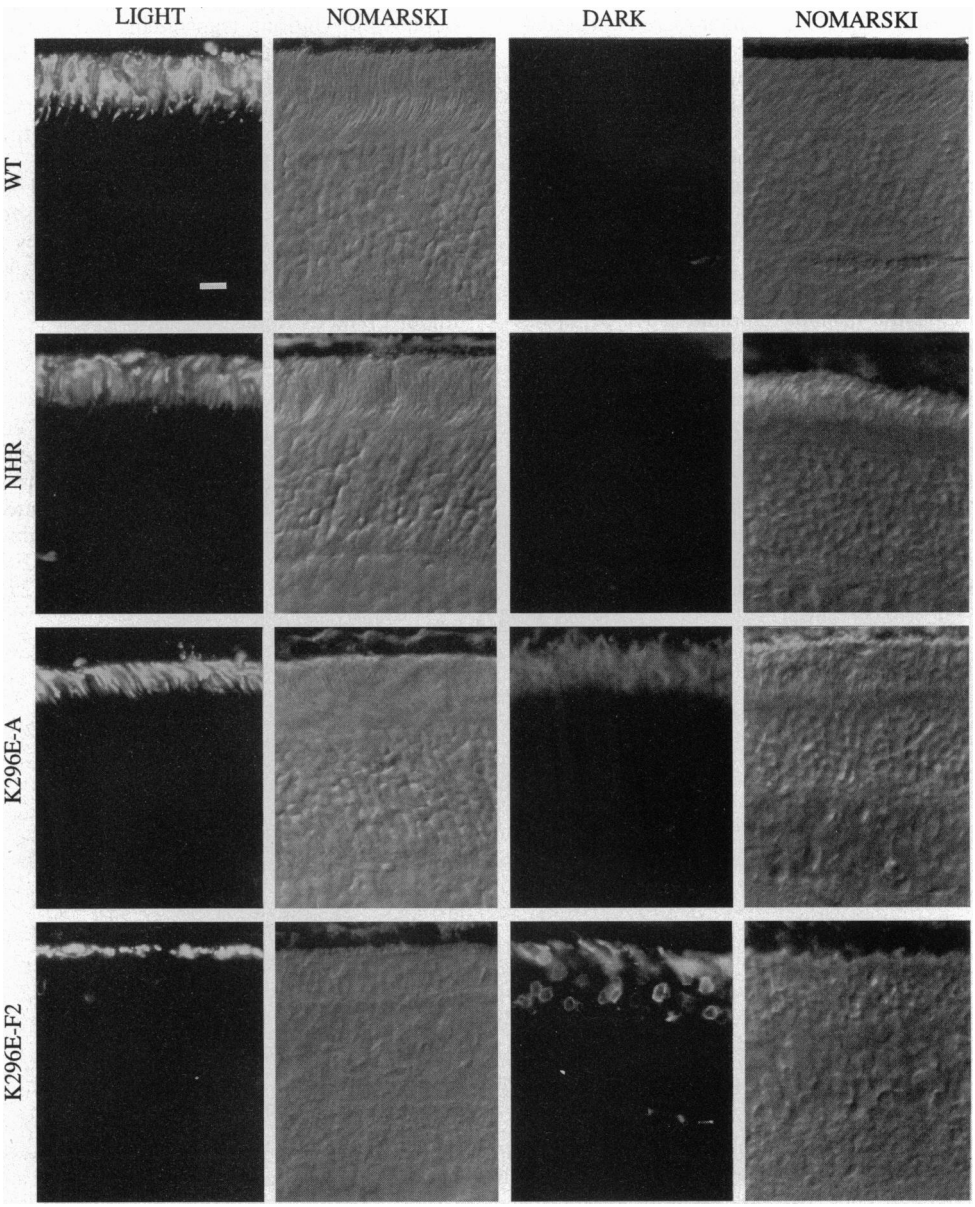

FIG. 2. Light-independent phosphorylation of opsin in transgenic retinas. Frozen sections were cut from dark- or light-adapted transgenic and control retinas (≈1 month of age) and stained with the antibody A11-82P. Nomarski images are shown for reference. (Bar = 10 μm.)

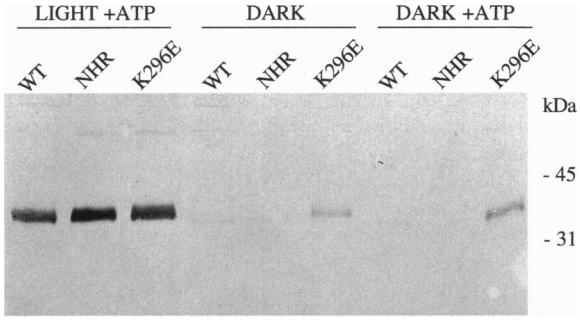

FIG. 3. Immunoblot probed with antibody A11-82P showing comigration of the reactive protein in K296E ROS with *in vitro* phosphorylated opsin. ROS protein (0.25 μg) was loaded in each lane. Light + ATP, ROS preparation incubated in the presence of 3 mM ATP and white light. Dark, incubated in the dark under the same conditions without ATP. Dark + ATP, incubated with 3 mM ATP in the dark under the same conditions.

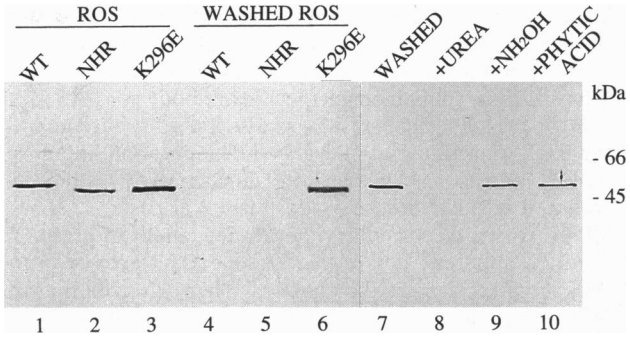

FIG. 4. Immunoblot showing stable interaction of K296E opsin with arrestin. The immunoblot of ROS proteins from dark-adapted retinas was probed with arrestin antibody. Lanes 1–6, 0.2 μg of protein was loaded; lanes 7–10, 0.1 μg of protein was loaded. Lanes 1–3, untreated ROS preparations; lanes 4–6, ROS preparations washed with hypotonic buffer; lane 7, hypotonically washed K296E ROS; lane 8, same as lane 7, washed again with 4.5 M urea; lane 9, same as lane 7, washed again with 1 mM hydroxylamine; lane 10, same as lane 7, washed again with 100 μM phytic acid.

remained in the membrane-bound fraction after hypotonic wash, indicating an association of arrestin with phosphorylated K296E opsin. This association was stable during prolonged incubations (hours) on ice and in storage at −20°C. Similar to arrestin bound to phosphorylated wt rhodopsin, arrestin bound to K296E opsin could be released by 4.5 M urea but not by 100 μM phytic acid (Fig. 4), which inhibits the binding of free arrestin to phosphorylated rhodopsin (21). Unlike complexes of arrestin and phosphorylated wt rhodopsin, however, complexes of arrestin and K296E opsin were not disrupted by washing with 1 mM hydroxylamine. Hydroxylamine removes all-*trans*-retinal from photoactivated wt rhodopsin, which in turn triggers the release of bound arrestin from phosphorylated rhodopsin (22). This observation was expected since K296E opsin is not bound to a chromophore. It also confirms that arrestin was indeed bound to K296E opsin instead of phosphorylated wt rhodopsin that might have been accidentally bleached during the preparation of ROS.

Light-Independent Activation of Transducin Could Be Demonstrated Only After Arrestin Removal and Dephosphorylation of Opsin. Transducin activation by dark-adapted ROS membranes was assayed in a reconstituted system using hypotonically washed ROS and separately using ROS membranes that had been further treated by urea-stripping and dephosphorylation with protein phosphatase 2A. Washed ROS membranes from K296E or control retinas did not activate transducin in the dark (Fig. 5*A*). However, after urea-stripping and dephosphorylation, transducin activation was detected in K296E ROS in the dark but not in NHR ROS treated identically (Fig. 5*B*). Confirmation that dephosphorylation had occurred was provided by the observation that dephosphorylated K296E opsin did not react with antibody A11-82P on immunoblots (not shown).

Dark Rearing of Transgenic Mice Did Not Prevent Degeneration. K296E F-2 line mice (n = 7) raised in the dark since birth were tested at 37–38 days of age by ERG. All showed reduced a- and b-wave amplitudes, with b-wave amplitudes at 286 ± 55 μV, which fell within the range of F-2 mice raised under cyclic lighting.

DISCUSSION

K296E opsin is a member of a group of constitutively active mutant opsins involving the chromophore binding site (6, 23) and is of particular interest because it is a known cause of dominant RP (5, 24). These mutants activate transducin *in vitro* with activities comparable to that of light-activated wt rhodopsin. It was shown recently that K296E opsin is not phosphorylated by rhodopsin kinase *in vitro* (25). Since phosphor-

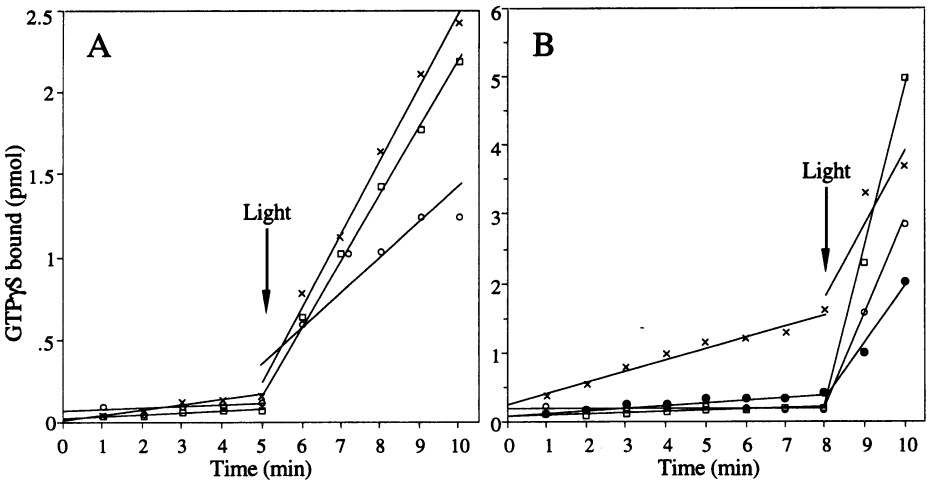

FIG. 5. Constitutive activity of K296E opsin was detectable only after removal of arrestin and dephosphorylation. Arrows indicate when light was turned on. (*A*) *In vitro* transducin activation assay using hypotonically washed ROS membranes. Rhodopsin was present at 5 nM in the reaction mixture. ○, wt; □, NHR; x, K296E. (*B*) *In vitro* transducin activation assay using urea-stripped ROS membranes with or without phosphatase treatment. Rhodopsin was present at 40 nM in the reaction mixture. NHR (□) and K296E (●) ROS without phosphatase treatment; NHR (○) and K296E (x) ROS treated with phosphatase $2A_1$.

ylation of photo-activated rhodopsin by rhodopsin kinase is a key step in the inactivation of phototransduction (26), K296E opsin was predicted to activate the phototransduction cascade continuously *in vivo*. Assuming that half of the opsin in the photoreceptor cells of patients with RP is the mutant, this would hypothetically create a situation analogous to continuous exposure to very bright light, which could readily explain the demise of the photoreceptor cells.

This hypothesis predicts that the K296E transgenic mice would display a light-adapted ERG even in the dark. However, the prolongation in implicit times seen in these mice, which resemble those in human patients with early RP (27), contrast with the shorter b-wave implicit times seen in ERGs of light-adapted normal individuals (28). Furthermore, assays for transducin activation using purified ROS indicate that there is no light-independent activation of the phototransduction cascade *in vivo*. We found this to be due to inactivation of K296E opsin by phosphorylation and arrestin binding. Unlike phosphorylated metarhodopsin II, however, K296E opsin appears to be permanently bound to arrestin. Since arrestin prevents opsin dephosphorylation by protein phosphatase 2A (29), it is likely that K296E opsin would be stably phosphorylated *in vivo*.

It is interesting to note that K296E opsin is phosphorylated *in vivo*; yet this mutant opsin reportedly is not phosphorylated by rhodopsin kinase *in vitro* (25). It may be that K296E opsin is phosphorylated by protein kinase C *in vivo* (30). Alternatively, rhodopsin kinase may phosphorylate K296E opsin at a much reduced rate, such that it is below the detection limit in the *in vitro* experiment. The lower activity toward K296E opsin would be largely offset *in vivo* by a presumed lack of turnovers of phosphorylated K296E opsin. Finally, K296E opsin in the disc membrane may become phosphorylated by rhodopsin kinase activated by neighboring bleached wt rhodopsin when mice are raised under cyclic lighting.

Our study establishes that constitutive activation of phototransduction by K296E opsin does not play a direct role in photoreceptor death. Could it be then that K296E opsin causes cell death indirectly by depleting or sequestering cellular components required to shut it off—e.g., arrestin? In *Drosophila*, a substantial reduction in arrestin causes photoreceptor death, but cell death can be prevented by avoiding light exposure (31). In our study, dark-rearing did not prevent or significantly delay cell death. This suggests that arrestin depletion is not the cause of cell death in K296E mice, although we cannot exclude a possible reduction of available arrestin and, consequently, changes in photoreceptor inactivation kinetics. Our current data do not point to a specific biochemical defect that leads to photoreceptor degeneration. However, instability of K296E opsin (6) or a novel pathogenic mechanism of excessive phosphorylated opsin, as has been implicated in *Drosophila* (32), are interesting possibilities.

Some observations on stationary night blindness are also inconsistent with the equivalent light hypothesis. At least two rhodopsin mutants causing stationary night blindness in humans, Ala-292 → Glu and Gly-90 → Asp, have been shown to activate transducin constitutively *in vitro* when not bound to a chromophore (33, 34). *In vivo* these mutant opsins cause sufficient light adaptation to eliminate the rod ERG; yet the photoreceptor cells survive for years. In comparison, among mouse models for RP carrying mutant opsin alleles, including the K296E model, none has shown such a degree of endogenous light adaptation.

In summary, the available data are inconsistent with the hypothesis that continuous equivalent light exposure is a generalized mechanism of cell death underlying dominant rhodopsin mutations. The mechanism of cell death caused by these mutations remains to be elucidated, for which the study of *in vivo* models should be an important component.

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