A Comparison of the Efficiency of G Protein Activation by Ligand-Free and Light-Activated Forms of Rhodopsin

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ABSTRACT Activation of the photoreceptor G protein transducin (G_t) by opsin, the ligand-free form of rhodopsin, was measured using rod outer segment membranes with densities of opsin and G_t similar to those found in rod cells. When GTPγS was used as the activating nucleotide, opsin catalyzed transducin activation with an exponential time course with a rate constant k_{act} on the order of 2×10^{-3} s^{-1}. Comparison under these conditions to activation by flash-generated metarhodopsin II (MII) revealed that opsin- and R*-catalyzed activation showed similar kinetics when MII was present at a surface density $\sim 10^{-6}$ lower than that of opsin. Thus, in contrast to some previous reports, we find that the catalytic potency of opsin is only $\sim 10^{-6}$ that of MII. In the presence of residual retinaldehyde-derived species present in membranes treated with hydroxylamine after bleaching, the apparent k_{act} observed was much higher than that for opsin, suggesting a possible explanation for previous reports of more efficient activation by opsin. These results are important for considering the possible role of opsin in the diverse phenomena in which it has been suggested to play a key role, such as bleaching desensitization and retinal degeneration induced by continuous light or vitamin A deprivation.

INTRODUCTION

Activation of G proteins by ligand-free receptors of the heptahelical family may be expected to play an important role in setting the background level of signaling above which the responses triggered by extracellular stimuli must be detected. For β-adrenergic receptors, results from overexpression experiments suggest that high levels of ligand-free receptor can generate signals equivalent to low levels of hormone (Samama et al., 1993; Milano et al., 1994). In the case of the photon receptor, rhodopsin, an important mechanism for allowing sufficient signal-to-background ratios for single photons to be detected is the action of 11-*cis*-retinal as an antagonist. This covalently attached ligand keeps rhodopsin locked into an inactive conformation in the dark, with G protein-activating activity so low that it has never been detected biochemically. Because spontaneous activation of the photoreceptor G protein transducin (G_t) occurs with a rate constant of 10^{-5}-10^{-4} s^{-1} (Fawzi and Northup, 1990; Ramdas et al., 1991; Matesic and Liebman, 1992), and the photoactivated form of rhodopsin, metarhodopsin II (MII), can catalyze G_t activation at reported rates of 10^3 to 10^4 s^{-1} (Vuong et al., 1984; Kahlert and Hofmann, 1991; Pugh and Lamb, 1993; this work), the signal-to-background ratio at this stage of the phototransduction cascade is greater than 10^7. (In this paper, MII refers to metarhodopsin II, R* is used to refer collectively to all rhodopsin photoproducts containing all-*trans*-retinal, and R is used to refer collectively to all forms of rhodopsin, R* and opsin.) In contrast to the extremely low activity of rhodopsin, several reports have described measurable activation of G_t by opsin, the apoprotein form of rhodopsin lacking covalently attached retinal (Okada et al., 1989; Fawzi and Northup, 1990; Cohen et al., 1993; Cornwall and Fain, 1994; Surya et al., 1995; Jager et al., 1995; Matthews et al., 1996). Although this species is not expected to be present at significant levels in dark-adapted rod cells, it may accumulate as a result of continuous illumination or bright flashes, and may also result from vitamin A deprivation, or mutations in rhodopsin preventing retinal binding (Dowling and Wald, 1958; Noell and Albrecht, 1971; Keen et al., 1991; Sullivan et al., 1993; Fain and Lisman, 1993). It has been suggested that it may play an important role in physiologically important phenomena, including bleaching desensitization (Dowling, 1960; Rushton, 1961; Pepperberg et al., 1978; Jin et al., 1993) and some forms of retinal degeneration (Fain and Lisman, 1993).

Unfortunately, the role of opsin in these processes, and possibly in setting signal-to-background limits for phototransduction in vivo, has been difficult to assess because of wide variations in the estimates of the potency with which opsin can catalyze nucleotide exchange by G_t (Table 1). In early work, Okada et al. (1989) reported G_t activation by postmetarhodopsin II (MII) photoproducts, presumably opsin, that appeared to be nearly as efficient as MII. More recent studies indicated that G_t activation by opsin is slower than activation by MII, but in one case (Surya et al., 1995) only by a factor of ~30. However, electrophysiological measurements of intact, fully bleached rod cells detected residual activation of the signal transduction cascade at a level about a million-fold lower than for the fully active photoproducts (Cornwall and Fain, 1994). Because activation of a single receptor may be sufficient to elicit a cellular response in the rod cell (Hecht et al., 1942; Baylor et al.,

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TABLE 1 Experimental comparisons of opsin and R* activation of G_t

Study	Opsin k_{act}/R* k_{act}	Assay for G_t activation	Removed retinal oxime
Okada et al. (1989)	$\sim 8 \times 10^{-1}$	GTPase assay	N
Cohen et al. (1993)	$\sim 1 \times 10^{-1}$*	filter binding	Y#
Fawzi and Northup (1990)	5.8×10^{-2}§	filter binding	N
Surya et al. (1995)	3.4×10^{-2}	filter binding	Y
Jager et al. (1995)	4×10^{-3}	G_t fluorescence	N‖
Cornwall and Fain (1994)	$\sim 10^{-6}$	PDE¶	Y**
This study	2×10^{-6}	PDE	Y
Fukada and Yoshizawa (1981)	Not detectable##	PDE§§	Y
Palczewski et al. (1994)	Not detectable‖‖	G_t fluorescence	N

* For assays conducted at acidic pH (6.1). No detectable opsin activity at neutral pH.
# Opsin expressed in COS cells in the absence of any retinal.
§ Opsin activity compared to opsin regenerated with 9-cis retinal.
¶ Electrophysiological measurements of cGMP-gated currents in rod cells. PDE-derived changes in the current were monitored by using Li^+ to inhibit guanylyl cyclase.
‖ Primarily used NH_2OH-washed membranes in which retinal-oxime was not removed. Some experiments used fully bleached membranes treated with hydrazide AvidGel Ax to remove all-*trans* retinal.
** In intact rods, all-*trans* retinal removed through reduction by retinol dehydrogenase.
Opsin activity not detectable. Residual PDE activity was less than in background dark-adapted membranes; however, activity less than 1×10^{-2} s^{-1} would likely not be detectable under the assay conditions described.
§§ Followed [^{3}H]cGMP hydrolysis by thin-layer chromatography.
‖‖ Opsin activity not detectable at pH 6.0 in 10 mM NH_2OH. Opsin k_{act}/R* k_{act} detection limit of 4×10^{-3}.

1979), and because these processes might be expected to apply to other G-protein coupled receptors in addition to rhodopsin, it is important to determine the actual activity of the ligand-free opsin apoprotein. Inspection of Table 1 makes it clear that this issue is not settled and bears reexamination.

In examining this question, an important issue is the appropriate environment in which to study G_t-receptor (opsin or R*) interactions. In intact rod cells, these interactions occur at the surface of disk membranes on which rhodopsin is found at surface densities of ~25,000 μm^{-2} (reviewed in Liebman et al., 1987), and G_t is found at about one-tenth that density (Hamm and Bownds, 1986). Studies of G_t-R* interactions in detergent solution (Nakayama and Khorana, 1991), in vesicles reconstituted with purified rhodopsin by detergent dialysis (Tyminski and O'Brien, 1984; Wessling-Resnick and Johnson, 1987), or in very dilute membrane suspensions (Zhukovsky et al., 1991), have been very useful for comparing properties of rhodopsin with those of mutant forms, and for establishing the relative roles of rhodopsin itself, and the other membrane components present in disks. Such studies can also take advantage of the simplified treatment of kinetics that is in principle possible with enzyme (R* or opsin) and substrate (G_t) in dilute solution rather than densely packed at a solution-membrane interface. Nonetheless, we have chosen to make the comparison on disk membranes to facilitate understanding the possible role of opsin in functioning rod cells.

An important tool in studying the function of opsin, rhodopsin, and their various photochemical forms has been treatment with hydroxylamine (NH_2OH). This treatment is an efficient and widely used mechanism for accelerating the conversion of bleached rhodopsin to opsin. NH_2OH attacks the Schiff base formed between the chromophore and either Meta I or MII (Dartnall, 1967; Brin and Ripps, 1977; Ratner et al., 1981), but not that formed by dark-adapted rhodopsin, producing opsin and retinal-oxime. Opsin can then be made to associate with a variety of chromophore analogs (Pepperberg et al., 1978; Corson et al., 1990; Jin et al., 1993). In the studies summarized in Table 1, there has been considerable variation in the methods used (if any) to remove retinal oxime or other retinal derivatives from opsin preparations, and there have been conflicting reports on whether retinal oxime affects "opsin" activity. Therefore, in this work we have focused considerable attention on the effects of increasing hydroxylamine concentrations and of treatments to remove retinal oxime and other retinal-derived species, on apparent G_t activation by "opsin."

MATERIALS AND METHODS

Buffers

The buffers used were the following: ROS buffer, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 30 mM NaCl, 60 mM KCl, 1 mM dithiothreitol (DTT), solid phenylmethylsulfonyl fluoride (PMSF), pH 7.5; pH assay buffer 1, 20 mM MOPS, 2 mM $MgCl_2$, 150 mM KCl, 1 mM DTT, solid PMSF, pH 8.1; pH assay buffer 2, 25 mM Tris, 2 mM $MgCl_2$, 150 mM KCl, 1 mM DTT, solid PMSF, pH 8.1; pH assay buffer 3, 10 mM Tris, 2 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, solid PMSF, pH 8.1; hypotonic buffer, 1 mM MOPS, 0.1 mM $MgCl_2$, 7.5 mM KCl, 1 mM DTT, solid PMSF, pH 7.5. All buffers were filtered through 0.2 μm nitrocellulose filters immediately before use. Hydroxylamine solutions were prepared as NH_2OH-HCl, with pH adjusted to 8.0 with HCl; in the text and figures, "NH_2OH" refers to NH_2OH-HCl.

Rod outer segments and protein preparation

Rod outer segments (ROSs) were isolated from frozen bovine retinas (J. A. Lawson Packing, Lincoln, NE) under dim red light with the sucrose

gradient method (Papermaster and Dreyer, 1974). The final ROS pellet was resuspended in ROS Buffer, aliquoted, and stored at −80°C.

Formal ROS membrane concentrations (containing rhodopsin, R*, or opsin) are expressed in terms of total rhodopsin (R), as assessed before bleaching or other treatment by absorbance measurements (500 nm) in the presence of 1.5% (w/v) *N,N*-dimethyldodecyl-amine-N-oxide (LDAO).

PDE (cGMP phosphodiesterase) and G_t were prepared as crude extracts from bleached ROSs. ROSs were bleached and diluted to 15–30 μM R in hypotonic buffer. GTP was added in a concentration approximately equimolar to that of R. The membranes were sedimented by centrifugation immediately for 5 or 10 min at 70,000 × *g* in a TL-100 tabletop ultracentrifuge at 4°C, and the supernatant containing PDE and G_t was removed. Free GTP was removed from the PDE/G_t supernatants by repeated dilution/concentration cycles with GTP-free hypotonic buffer in a Centricon-30, resulting in at least an 8000-fold dilution of GTP. The concentrated proteins were stored in 40% glycerol at −20°C. Alternatively, PDE and G_t were isolated separately by first washing ROS membranes with hypotonic buffer alone (to extract PDE) and subsequently washing with hypotonic buffer plus GTP (to extract G_t).

Preparation of opsin-containing membranes

Membranes previously stripped of PDE and G_t were resuspended in buffer (either pH assay buffer 1 or 2) plus 100 mM NH_2OH adjusted to pH 8.0, homogenized by repeated passage through a 23-gauge needle, and incubated on ice under a tungsten light, for 15 min before being pelleted again. The membranes were then washed in buffer alone. Both washes (NH_2OH, followed by buffer alone) were repeated once more. Finally, membranes were resuspended to original concentrations in buffer and either used immediately or frozen at −80°C. Control membranes containing light-activated rhodopsin (composed of MII and other photoproducts, collectively referred to here as R*) were washed identically, except that no NH_2OH was included. Previous studies have shown that for comparable concentrations of R*-containing membranes, the second-order rate constant for the reaction

$$NH_2OH + R^* \rightarrow \text{retinal oxime} + \text{opsin} \qquad (1)$$

is $1.45 \times 10^{-3}\ s^{-1}\ mM^{-1}$ when G_t is bound, and is increased ~10-fold for membranes stripped of G_t (Hofmann et al., 1983). Therefore, under our conditions, at 100 mM hydroxylamine, the reaction should reach 99% completion within 32 s for any R* with residual G_t bound, and within 3.2 s for the majority of R* with no G_t bound. Hydroxylamine-mediated hydrolysis of rhodopsin-associated palmitate is not likely to occur at significant levels under our conditions (Morrison et al., 1991), and loss of palmitate would not be expected to effect greatly transducin activation (Karnik et al., 1993).

Removal of retinal oxime from membranes

Retinal oxime was removed by incubation with sonicated phosphatidylcholine vesicles (Stubbs et al., 1976). Egg phosphatidylcholine (~150–250 mg; Avanti) was dried under nitrogen to form a thin film on the base and sides of a 15-ml Corex tube. Residual solvent was removed under vacuum overnight. The lipids were resuspended in pH assay buffer 2 by light vortexing. The lipids were sonicated on ice for seven cycles of 2 min on, 1 min off with a microtip sonicator (Branson Sonifier Cell Disrupter 185, power setting 5). The lipid solution was stored at 4°C overnight, allowing the smallest vesicles to fuse. Large or multilamellar vesicles were removed by sedimentation at 232,000 × *g* for 2 h. NH_2OH-washed membranes were mixed at a 10-fold molar excess of vesicle lipid over ROS lipid and left at 4°C for times between 45 min and 18 h, as indicated. In some experiments 100 mM NH_2OH was included in the vesicle wash. The membranes were sedimented at 25,800 × *g*. The supernatants were removed and saved for spectral analysis, and the pellets were resuspended and stored at −80°C. UV/visible absorbance spectra (365 nm) were measured for samples of the pellet and supernatant in the presence of 1.5% (w/v) LDAO.

Regeneration with 11-*cis*-retinal

Dim red light or completely dark conditions, and an argon blanket were used for all procedures with 11-*cis*-retinal before flash activation. Crystals of 11-*cis*-retinal were dissolved in absolute EtOH to a final concentration of 8 mM. Membranes were incubated with retinal at a 4–10-fold molar excess of retinal to opsin. The mixtures were shaken at low speed on a vortexer for 15 min to 2 h at either room temperature or 4°C. Then the membranes were recovered by sedimentation at 70,000 × *g*. The membranes were resuspended in pH assay buffer 3 and kept on ice until use. Absorbance spectra of the resuspended membranes were measured in 1.5% (w/v) LDAO.

Assay for PDE activity

PDE activity was determined by following the changing pH due to proton release during cGMP hydrolysis as described (Liebman and Evanczuk, 1982; Malinski and Wensel, 1992). Assays were carried out in 300-μl volumes in 96-well microtiter plates. Washed ROS membranes were used as a source of R* or opsin, and as a surface for PDE activation; protein extracts were readded to reconstitute initial ROS protein concentrations. The pH of nucleotide samples was adjusted to minimize pH changes during nucleotide addition. For experiments with varying [NH_2OH-HCl], the samples were maintained at constant ionic strength by varying [NaCl], to avoid inhibition of PDE caused by increasing total ionic strength. The pH during the assays always ranged between 8.1 and 7.7. The assays were conducted with either MI-410 or MI-415 microelectrodes monitored by a Corning Ion Analyzer 250. The electrode potential was recorded both on a chart recorder and on a computerized acquisition system described previously (Angleson and Wensel, 1993). Derivative plots are not corrected for basal PDE activity, which was always less than 10% of the maximum G_t-dependent PDE activity.

Analysis of activation kinetics

Because the encounter between G_t and either opsin or R* occurs through a process of two-dimensional diffusion on the membrane surface, treatment with the methods of classical solution enzymology is not appropriate. It can be shown that for reasonable estimates of G_t and R* diffusion coefficients, diffusion-limited activation of G_t by R* should occur with a time-dependent rate constant (Lamb and Pugh, 1992); however, in our experimental conditions, we have found that slow activation of G_t by either opsin or low mole fractions of R* is fairly accurately described by a single exponential. Therefore, for purposes of comparison, we have chosen to define the rate constant characterizing the exponential approach to complete G_t activation as k_{act}:

$$G_t^*(t) = G_{tmax}^*(1 - \exp[-k_{act}t]) \qquad (2)$$

The value of k_{act} may be expected, a priori, to vary as a function of the surface density of opsin or R* and G_t, as well as of the functional state of opsin (opsin versus R* versus rhodopsin). Therefore we used the surface densities of rhodopsin or opsin and G_t naturally found in our ROS preparations in all experiments. Because GDP release is normally the rate-limiting step in transducin nucleotide exchange (Ramdas et al., 1991), while binding of GTP or GTPγS to nucleotide-free G_t occurs with a rate constant of $7.6\ \mu M^{-1}\ s^{-1}$ under our conditions (Angleson and Wensel, 1993; Kahlert and Hofmann, 1991), in the range of nucleotide concentrations used here (32–165 μM), k_{act} is not expected to depend on nucleotide concentration. We tested this expectation by varying GTP concentration over a fourfold range, and confirmed that there was no detectable dependence of k_{act} on nucleotide concentration.

G_t activation kinetics were monitored by continuous recording of PDE activity, which under our conditions is well established to be directly proportional to G_t activation (Ramdas et al., 1991; Angleson and Wensel, 1993; Malinski et al., 1996). The value of k_{act} was determined by nonlinear least-squares fitting of single exponential curves to the first derivative with

respect to time of the voltage output of the pH electrode monitoring cGMP hydrolysis:

$$PDE^* = PDE^*_{max}(1 - \exp[-k_{act}\, t]) \quad (3)$$

where PDE* represents the increase in PDE activity ($-$d[cGMP]/dt, directly proportional to d(voltage)/dt), above the basal level observed before G_t activation. PDE^*_{max} represents the maximum G_t-dependent PDE activity in the sample upon activation of all G_t by GTPγS.

For experiments involving GTP, hydrolysis of GTP inactivates G_t and PDE, so while the time course of PDE activity is still exponential, the maximum level of PDE activity is set by the steady-state balance between activation and inactivation, and both the activation rate constant (k_{act}) and the GTP hydrolysis rate constant (k_{inact}) contribute to the approach to steady state:

$$mpos;lPDE^*(t) = PDE^*_{max}(k_{act}/(k_{act} + k_{inact})) \cdot (1 - \exp[-(k_{act} + k_{inact})t]) \quad (4)$$

where PDE^*_{max} here is the value observed in the absence of hydrolysis (i.e., with GTPγS). Under the conditions of our assays, k_{inact} has been found to fall in the range of 0.02–0.05 s^{-1} (Angleson and Wensel, 1993, 1994), and so is expected to dominate the kinetics of approach to steady state if $k_{act} \ll 0.02\ s^{-1}$, as it consistently was in our opsin experiments. Changes in k_{act} are manifested primarily as changes in the steady-state PDE* activity:

$$PDE^*_{steady\text{-}state}/PDE^*_{max} = k_{act}/(k_{act} + k_{inact}) \quad (5)$$

Under these conditions, $k_{act} + k_{inact}$ can be determined from fitting the approach to steady state to Eq. 4, giving, along with substitution of the measured steady-state PDE activity into Eq. 5, two equations that can be solved for the two unknowns, k_{act} and k_{inact}.

Flash activation

Flash experiments were carried out using either untreated ROSs or ROSs washed and reconstituted (including treatment with NH_2OH and vesicles plus NH_2OH), just as with the bleached ROSs used to prepare opsin, except that procedures were all carried out in the dark. PDE assays on dark ROSs were initiated by flashes of light from a battery-powered electronic flash lamp passed through a 546-nm interference filter. Fractional photoisomerization of rhodopsin was determined by spectrophotometry in 1.5% LDAO after flashing, and attenuation varied by calibrated neutral density filters. As in the experiments with opsin, GTPγS was present at a concentration of 32 μM.

RESULTS

G_t activation by NH_2OH-washed membranes and GTP

When GTP was added to membranes treated successively with hydroxylamine and vesicles to generate opsin from R* and remove retinal oxime, fairly robust activation of G_t, as revealed by PDE activity, was observed (Fig. 1). The maximum level of activity observed under these conditions was much lower than for a control sample not treated with hydroxylamine, and the time course of approach to steady state was much slower in the opsin-containing, than in the R*-containing membranes. Analysis of the time course of PDE activation, and the steady-state level of activity, yielded a value for k_{act} of $3.0 \times 10^{-3}\ s^{-1}$ for opsin. The average k_{act} from four assays was $3.8 \pm 0.55 \times 10^{-3}\ s^{-1}$. The side-by-side comparison in Fig. 1 with R*-containing

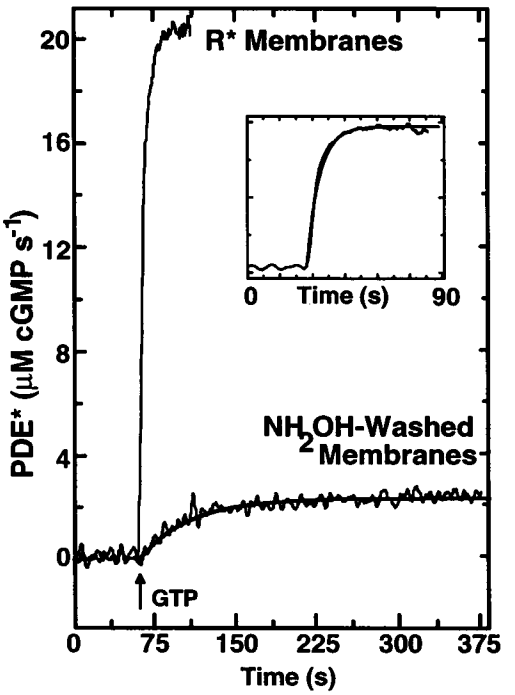

FIGURE 1 G_t activation by GTP and opsin. NH_2OH-washed and control R* (buffer-washed) membranes were incubated with a sevenfold molar excess (vesicle lipid/ROS lipid) of PC vesicles for 12 h. The vesicles were washed away, and membranes (7.5 μM R) were reconstituted with G_t and PDE (to the same levels present on the membranes initially) and assayed for PDE activity stimulated by the addition of GTP (165 μM), using 2 mM cGMP and pH assay buffer 3. The signal was integrated digitally for 0.2 s, with 50-ms sampling. Traces are derivatives of pH recordings calculated over 2.6-s windows at 0.2-s intervals after first-order smoothing over 2.6-s intervals, and are not corrected for basal PDE activity. The smooth lines are fits to the steady-state rate constant = $k_{act} + k_{inact}$. For the NH_2OH-treated membranes (*lower curve*) $k_{act} + k_{inact} = 0.024\ s^{-1}$, and k_{act} was calculated as described in the text to be $3 \times 10^{-3}\ s^{-1}$. The fit to the R* curve is shown on an expanded time scale in the inset and is $k_{act} + k_{inact} = 0.214\ s^{-1}$.

membranes not treated with hydroxylamine provides a useful positive control for the assay, and establishes the value of PDE^*_{max} for use in Eq. 5. However, it does not provide an accurate measure of k_{act} for R*, as k_{act} is not limiting in this case. Conditions where k_{act} is limiting were established in flash experiments described below.

G_t activation by NH_2OH-washed membranes and GTPγS

When GTP was used, k_{act} had to be inferred indirectly from steady-state PDE activity, because of the dominance of the kinetics by k_{inact}. To verify k_{act} more directly and more accurately, we initiated activation with GTPγS, and used the exponential time course of PDE activation, rather than the steady-state value, to determine a k_{act} value for membranes treated to generate opsin and remove retinal oxime, as in Fig. 1. The k_{act} determined this way (Fig. 2, *lower trace*) was $1.8 \times 10^{-3}\ s^{-1}$, reasonably consistent with the value obtained with GTP. The average k_{act} (± standard

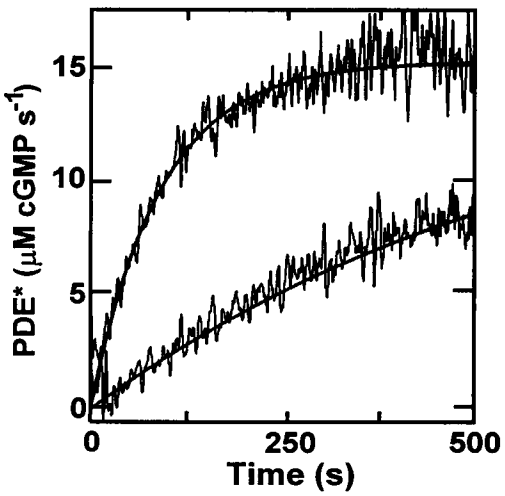

FIGURE 2 Activation by GTPγS in opsin-containing membranes. *Upper trace*: Membranes generated by washing bleached membranes twice with hydroxylamine, and assayed without added hydroxylamine. *Lower trace*: Membranes prepared by washing bleached membranes twice with hydroxylamine, followed by vesicle washing to remove retinal-oxime, and assayed in the presence of 150 mM NH_2OH. Noisy traces are derivatives of pH electrode outputs. The smooth lines show the single exponential curves fit to the data to derive k_{act} values. Calculated k_{act} values were 1.1×10^{-2} s^{-1} (*upper smooth line*) and 1.8×10^{-3} s^{-1} (*lower smooth line*).

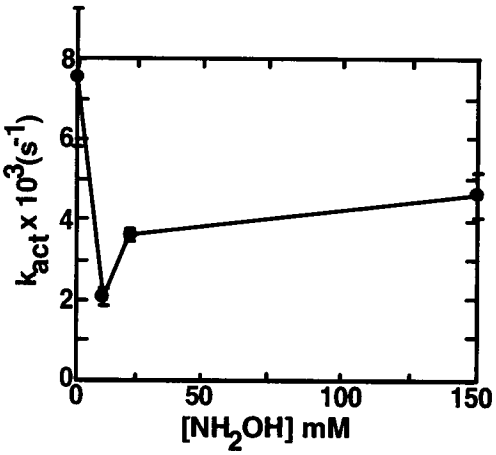

FIGURE 3 NH_2OH-sensitive component in NH_2OH-washed membranes. PDE assays of NH_2OH-washed membranes (10 μM R, no vesicle washing) reconstituted with G_t and PDE were conducted in pH assay buffer 2 (with NH_2OH-HCl and/or NaCl substituted for KCl) and initiated with 32 μM GTPγS. NH_2OH-HCl was included in the assays at the indicated concentrations, and the assay mixture was kept isotonic by varying NaCl ([NH_2OH-HCl] plus [NaCl] = 150 mM). Membranes were allowed to incubate with NH_2OH for 4 min before the addition of cGMP (6 mM) and GTPγS. Points are averages of duplicate or triplicate assays ± standard deviation.

deviation) for membranes treated with successive hydroxylamine and vesicle plus hydroxylamine washes for 21 samples from six different preparations was $1.76 \pm 0.85 \times 10^{-3}$ s^{-1} when assayed with GTPγS.

Increased activating potency in the presence of residual retinal or retinal-oxime

When membranes were washed with hydroxylamine, but not with vesicles, a faster k_{act} was consistently observed (Fig. 2, *upper trace*). We therefore studied the dependence of k_{act} on hydroxylamine concentration, and on the details of the washing procedures used to remove retinal oxime.

Fig. 3 shows a comparison of k_{act} values observed for membranes washed with hydroxylamine, but not with vesicles, and then assayed with GTPγS in the presence of various hydroxylamine concentrations. The addition of hydroxylamine to moderate levels (10 mM) led to a reduction in k_{act} to a minimum value of 2.1×10^{-3} s^{-1}, very similar to the values observed when vesicle washing was used to remove retinal oxime, whereas higher concentrations (20–150 mM) actually decreased k_{act} somewhat less. The observation that some of the apparent "opsin" activation is sensitive to hydroxylamine addition is consistent with previous observations (Palczewski et al., 1994). The simplest explanation is that when excess hydroxylamine is removed, some of the retinal-oxime reverts by hydrolysis to all-*trans* retinal, which then associates with opsin; readding hydroxylamine converts the all-*trans* retinal back to retinal-oxime. The noncovalent complex of all-*trans* retinal plus opsin is known to have much greater potency in G_t activation than opsin has (Jager et al., 1995; Palczewski et al., 1994). Retinal-oxime itself plus opsin is not likely to form an active receptor, as studies of the potency with which opsin is phosphorylated suggest that retinal-oxime is too large to fit in the chromophore binding site (Buczylko et al., 1996). This explanation is supported by the disappearance of almost all of the hydroxylamine sensitivity when membranes were washed with vesicles to remove most of the retinal-oxime before assaying (Fig. 4 *A*). Fig. 4 *B* shows the results from multiple washes with vesicles, in which hydroxylamine was present during the washing procedure to help prevent opsin complexes with retinal from forming. The substantial decline in k_{act} after the first wash and the plateau reached with subsequent washes imply that the value of k_{act} observed after treatment with vesicles and hydroxylamine reported above (1.76×10^{-3} $s^{-1} \pm 0.85 \times 10^{-3}$ s^{-1}) is the most reliable estimate for the value characteristic of opsin, and that significantly higher values of k_{act} observed with less stringent washing reflect contributions by non-opsin species derived from residual retinal or retinal-oxime.

The efficiency of our vesicle treatment for retinal oxime removal as monitored by absorbance at 367 nm (Wald and Brown, 1953) was 85% for one wash. Varying the incubation time between 45 min and 18 h did not measurably affect the efficiency of retinal-oxime removal, suggesting that 85% represents an equilibrium partition coefficient under our conditions. If so, then after three washes we would expect to have removed 99.7% of the original retinal oxime, and after five washes to have reduced residual retinal oxime to less than 10^{-4} of its original amount, an estimate supported by the plateau in Fig. 4 *B*. The k_{act} for membranes

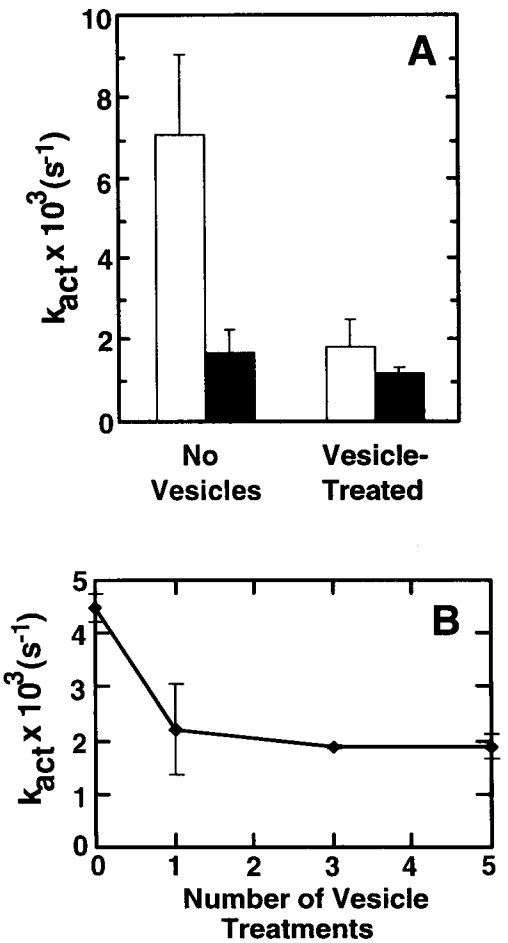

FIGURE 4 Complete removal of retinal-oxime eliminates NH_2OH sensitivity. (*A*) Treatment with sonicated PC vesicles reduces k_{act} and nearly eliminates NH_2OH sensitivity. Membranes were washed twice with pH assay buffer 2 plus 100 mM NH_2OH, followed by two washes of buffer alone. "Vesicle-treated" membranes were then washed with a sevenfold molar excess (vesicle lipid/ROS lipid) of PC vesicles (12 h incubation with vesicles), and "No vesicles" membranes were washed with buffer alone. Membranes were assayed in the presence of 150 mM NaCl (*empty bars*) or 150 mM NH_2OH (*filled bars*). pH assays were carried out on membranes (8 μM R) reconstituted with G_t and PDE extracts in pH assay buffer 2 (with NH_2OH-HCl or NaCl substituted for KCl). Assays were initiated by adding 32 μM GTPγS and cGMP to an initial concentration of 8 mM. For the "No vesicle" experiment with added NH_2OH (*filled bar*), $n = 3$, and $n = 4$ for all other conditions. (*B*) Simultaneous exposure to vesicles and NH_2OH results in the lowest k_{act} and complete elimination of NH_2OH sensitivity. Membranes were washed twice with pH assay buffer 2 plus 100 mM NH_2OH, followed by two washes of buffer alone before being treated the indicated number of times with a 10-fold lipid:lipid molar excess of PC vesicles and 100 mM NH_2OH simultaneously (45-min incubations). Assays were conducted in the same buffer, except that NaCl was substituted for KCl. Membranes (20 μM R) were reconstituted with PDE and G_t, and hydrolysis of 8 mM cGMP was monitored after the addition of GTPγS (32 μM). Assays were carried out with additional 20 mM NH_2OH-HCl, and the NaCl concentration was reduced to 130 mM to keep the assay conditions isotonic. After three vesicle/NH_2OH treatments, retinal-oxime levels were below our spectroscopic detection limits. Points are averages of duplicate or triplicate assays ± standard deviation.

washed five times with vesicles/NH_2OH was 1.89 (± 0.26) × 10^{-3} s^{-1}, and there was no significant sensitivity to inclusion of hydroxylamine in the assays.

Verification of protein integrity by regenerability with 11-*cis*-retinal

The decrease in k_{act} with increasingly stringent washing procedures could indicate that the opsin apoprotein is being damaged or lost. A diagnostic measure of opsin apoprotein that has retained a native-like fold is the ability to regenerate upon incubation with 11-*cis*-retinal a 500-nm absorbing species (rhodopsin) that exhibits light-dependent signal transduction. Regeneration with 11-*cis*-retinal on membranes that had been washed multiple times with NH_2OH resulted in restoration of efficient light-dependent activation of transducin (Fig. 5). Under the most stringent conditions, membranes that have undergone four vesicle plus NH_2OH washes regenerated at least 83% of the expected amount of rhodopsin, as determined by spectrophotometry. The loss of some regenerable protein is likely due to some loss of protein activity due to the somewhat harsh nature of the washes. Therefore, a 17% underestimation of opsin activity represents the upper limit on the possible error of our opsin activity measurements due to opsin denaturation.

Comparison to activation by R*

To compare G_t activation by opsin to activation by R* under conditions as similar as possible, we used dark-adapted ROS membranes with the same surface density of G_t and PDE, with or without exposure to the same washing procedure as in our opsin experiments, and generated R* in situ with dim flashes of light. Because R* activates G_t orders of

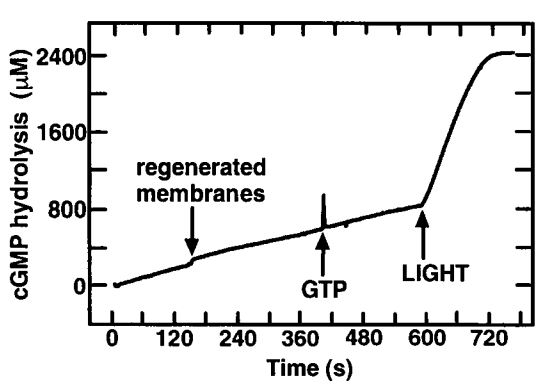

FIGURE 5 Regeneration of active rhodopsin in NH_2OH-treated membranes. NH_2OH-washed membranes were incubated with a fourfold molar excess of 11-*cis*-retinal (added in EtOH) for 2 h in the dark. The membranes were then washed once with pH assay buffer 1. The trace is of a pH assay carried out in the dark. Regenerated membranes (10 μM) were reconstituted with hypotonic extract containing PDE and G_t and assayed in pH assay buffer 1 with 3 mM NH_2OH-HCl. The assay was initiated with 2 mM cGMP and 60 μM GTP, and formation of MII was initiated by turning on the room lights.

magnitude faster than opsin does, we used very dim flashes and determined the fraction of photoexcitated rhodopsin at which the G_t activation kinetics coincided with those observed for opsin. Fig. 6 shows the results of light titrations, with opsin results included for comparison. As expected, at intensities bleaching less than 10^{-4} of total rhodopsin, the light dependence of k_{act} was essentially linear. Stringently washed, reconstituted dark ROS membranes and whole dark ROSs had similar activation kinetics. The closest agreement between R* activation kinetics and opsin activation kinetics was observed when R*/R was $\sim 1\text{–}2 \times 10^{-6}$, suggesting that opsin is on the order of 10^6-fold less efficient than R* at activating G_t.

Although our best estimate of k_{act} for opsin is 2×10^{-3} s^{-1}, it is worth estimating an extreme upper limit for k_{act}, allowing for the possibility that the multiple NH_2OH/vesicle washes or the presence of high [NH_2OH] reduced opsin activity by artifactual mechanisms rather than by removing retinal oxime or inhibiting its activation of opsin. Our most active "opsin" membranes, treated only with NH_2OH and light, and assayed without added NH_2OH, displayed a k_{act} of 10^{-2} s^{-1}, so this value represents an extreme upper limit for opsin's k_{act}. This value is equivalent to a flash activation with R*/R of $\sim 10^{-5}$ (Fig. 6).

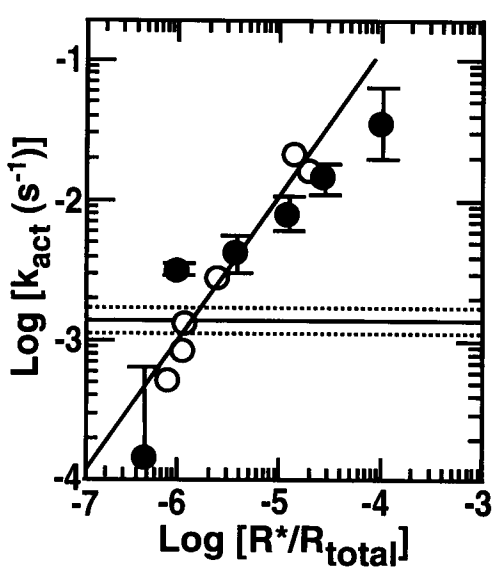

FIGURE 6 Comparison of G_t activation by opsin and flash-generated R*. Activation rate constants (k_{act}) were determined in standard pH assays (containing 32 μM GTPγS) measuring PDE-catalyzed hydrolysis of 8 mM cGMP. ROS membrane samples (all at 10 μM R) were reconstituted after treatment in the dark by washing as described in the text for preparing retinoid-free opsin from bleached membranes (washed ROS, ●), or unwashed dark-adapted ROS were used at 10 μM R (○). Opsin membranes prepared in parallel by identical treatment after bleaching were also assayed, and the result was plotted as the solid horizontal line, with error limits corresponding to ± standard deviation plotted as dashed horizontal lines. The predicted R* dependence of k_{act} (*solid line*) corresponds to a turnover number of 100 G_t s^{-1} R*−1 derived from equation 4 of Kahlert and Hofmann (1991), using our conditions as described in the text.

DISCUSSION

What is the most appropriate context for comparing the abilities of R* and opsin to activate G_t?

Visual transduction is greatly enhanced by the highly organized arrangement of transduction components within the rod cell. Receptor, G protein, and effector are all present at high formal concentrations (tens of micromolar to millimolar) and colocalized on the surface of disk membranes. It is in this context that any physiological function of opsin must be expressed. The convenience and apparent simplicity of assaying opsin or R* in dilute detergent solution or in very dilute membrane suspensions, and treating the results with classical enzyme kinetics, are balanced by difficulties in making direct comparisons to the situation in intact rods. Aside from the kinetic differences expected for reaction encounters resulting from predominantly two-dimensional as compared to three-dimensional diffusion, the critical kinetic parameters for this situation (e.g., half-saturating concentrations for G_t, k_{cat} for native MII) have yet to be determined, in contrast to the in-depth analysis that has been carried out for more intact membranes and natural ratios of G_t and rhodopsin (see Kahlert and Hofmann, 1991, and references therein). For these reasons, despite some technical drawbacks, we have chosen to compare opsin and R* catalytic potencies, using G_t bound to disk membranes, with rhodopsin (or opsin), G_t, and PDE all present at densities close to those occurring in intact rods. It would perhaps be even better to carry out the comparison using intact photoreceptors or retina, but such preparations are not amenable to the biochemical techniques used here.

Assays for G_t activation

As discussed below, some previous studies have failed to detect G_t activation by opsin, despite careful experimental procedures. Based on the results presented here, it seems likely that signal-to-noise or signal-to-background limitations inherent in the assays used in those studies present serious difficulties for comparing two catalysts whose activities differ by several orders of magnitude. For this reason we have used what is likely the most sensitive assay for G_t activation, stimulation of PDE activity. Although this assay is less direct than measurement of GDP release or GTPγS binding by G_t, it responds in a strictly linear way to G_t activation, and offers several advantages: 1) It is extremely sensitive because of amplification resulting from the catalytic activity of PDE. 2) It is easy to record a continuous trace on a subsecond time scale for accurate kinetic analysis. 3) It features built-in specificity (only transducin activates PDE in a GTP- or GTPγS-dependent way). In contrast, direct measurements of GTPγ^{35}S-binding suffer from background due to non-G_t proteins that bind GTPγS, covalent thiophosphorylation reactions (Wieland et al., 1991, 1993;

Malinski et al., 1996), and technical difficulties with filter washing that lead to high and varying background signal in some cases, and interfere with rapid and accurate kinetic measurements. There are also some disadvantages to our PDE assay. It requires the reconstitution of three separate components in the pathway, the membranes, G_t, and PDE, and the biochemical manipulations involved can lead to decreased membrane binding and coupling of $G_t\alpha$ and PDE, or to increased basal PDE activity. All of the experiments reported here were carried out with preparations in which basal PDE activity was less than 10% of the maximum level induced by G_t, and in which PDE stimulation by G_t was comparable to that observed before washing and reconstitution.

Comparison to other measurements of activation by R*

At a flash intensity giving rise to R*/R = 10^{-6}, k_{act} is $\sim 10^{-3}$ s^{-1}. Because there are about one-tenth as many G_t molecules as R in the sample, this corresponds to each R* activating $\sim$100 G_t each second at early times after the flash; the best fit for all of our flash data corresponds to 76 G_t per R* s^{-1}. This turnover number is in reasonable agreement with the results of Kahlert and Hofmann (1991), when the temperature and GTPγS concentration are taken into account. Specifically, their equation 4, which accounts accurately for light scattering measurements of G_t activation in permeabilized bovine ROS, predicts a turnover number in the range of 89–129 s^{-1} at 23°C and 32 μM GTP, assuming an initial G_t density of 3000 μm^{-2}. This range corresponds to the range 0.05–0.1 in their parameter α, which represents the probability that a collisional encounter of G_t and R* will lead to G_t activation. The value of α is much lower in permeabilized ROSs than in intact retina, where it approaches unity; however, it is possible that whatever lowers the G_t-R* coupling efficiency of permeabilized (or, in our case, thoroughly disrupted) versus intact ROSs may have the same effect on G_t-opsin coupling, so these values of α (0.05–0.1) are probably the most appropriate for comparing R* and opsin. It should be noted that these "turnover numbers" are not turnover numbers as the term is normally understood in classical enzymology, because G_t concentrations are not saturating. In addition, delays on the order of milliseconds introduced in PDE activation by the formation of metarhodopsin II and by diffusional encounters of PDE with activated G_t are not significant on the time scale of our experiments (Heck and Hofmann, 1993). Measurements with sheared amphibian ROSs indicated an even greater catalytic efficiency for R*, with a turnover number of 1000 s^{-1} at 500 μM GTP (Vuong et al., 1984). Thus our estimate of R* catalytic efficiency can be considered to be consistent with some previous results, and an underestimate as compared to others. It is certainly not an overestimate.

Comparison to other measurements of activation by opsin

Previous attempts to directly assess the ability of the opsin apoprotein to activate the visual transduction cascade have come to conflicting conclusions (Table 1). Measurements of transducin GTPase activity, used to compare activation by post-MII photoproducts prepared by treatment with hydroxylamine or prolonged incubation at 30°C, were reported to yield nearly identical potencies for opsin and MII (Okada et al., 1989). Because these measurements were carried out under conditions in which nucleotide exchange may not have been rate limiting, they are not likely to reflect accurately the relative catalytic potencies of opsin and MII. In addition, Table 1 makes it clear that measurements of opsin activation, like those by Okada et al. (1989) taken in the presence of retinal oxime, tend to be higher. Another anomalously high estimate of opsin potency in G_t activation employed measurements of GTPγS binding to G_t (Surya et al., 1995) to determine a catalytic efficiency of opsin 1/30th of that of MII. It is not entirely clear why this result differs so strikingly from most others (see below); however, at least part of the discrepancy appears to be due to an underestimation of the catalytic efficiency of MII, and part to generation of opsin under conditions that may not quantitatively remove retinal oxime. However, in this previous study, the addition of exogenous retinal oxime (Surya et al., 1995) was reported not to enhance the catalytic activity of opsin.

In contrast, a study of PDE activation induced in the dark by mixing in membranes resulting from various treatments (Fukada and Yoshizawa, 1981) found essentially no activation induced by opsin, but significant activation induced by bleached membranes containing both opsin and a full complement of retinal oxime. Because the sensitivity of these measurements was not sufficient to detect G_t activation at the levels we observe for opsin, and because they did detect significantly more activation potency in the presence of retinal oxime, these results (Fukada and Yoshizawa, 1981) are consistent with ours.

A more recent study (Jager et al., 1995) observed a tenfold lower opsin/R* activity ratio (1/250) than did the other recent report (Surya et al. 1995), despite not having gone to great lengths to remove all retinal oxime. Importantly, this work (Jager et al., 1995) confirmed earlier work (Hofmann et al., 1992) indicating that all-*trans* retinal and opsin form a complex distinct from MII that has much more activity than opsin, offering a possible explanation for the effects of residual retinal oxime.

A study employing opsin expressed in COS cells avoided the problem of residual retinal products (e.g., retinal oxime) and demonstrated measurable activation of G_t by opsin (Cohen et al., 1993). However, the necessary use of very dilute plasma membranes with low opsin densities and the low pH in those experiments make quantitative comparison to our results difficult: opsin accelerated G_t nucleotide exchange two- to threefold over the spontaneous rate (which was elevated by the low pH), whereas opsin regenerated

with 11-*cis*-retinal and assayed in the light-accelerated nucleotide exchange 10- to 15-fold. No acceleration of nucleotide exchange by opsin was detectable at pH 6.7. Thus these experiments were consistent with ours in 1) verifying unequivocally that opsin can activate G_t, and 2) revealing that its potency in this activation is orders of magnitude lower than that of MII at pH values near the physiological range.

Perhaps the most relevant results to date concerning opsin activity, with respect to understanding its possible physiological role, were obtained from electrical recordings from isolated salamander rods (Cornwall and Fain, 1994; Matthews et al., 1996). These experiments demonstrated that under bleaching conditions, G_t and PDE activities persist, but at a level expected for activation by opsin at $<10^{-6}$ the efficiency of R* (Cornwall and Fain, 1994; Matthews et al., 1996). These studies concluded that under their conditions, bleached pigment, or opsin, likely activates G_t with 10^{-6} to 10^{-7} times the efficiency of R*, in good agreement with our biochemical results from bovine opsin.

Whether this level of activity is sufficient to account for many of the phenomena that have been attributed to opsin is uncertain. A number of studies have demonstrated a relationship between opsin concentrations and bleaching desensitization levels (Dowling, 1960; Rushton, 1961; Pepperberg et al., 1978). It has been proposed that opsin may mediate this loss of sensitivity by constitutively activating a fraction of the available G_t pool (Jin et al., 1993). Our measured opsin activity is consistent with measurements of sustained PDE activity in isolated cells experiencing bleaching desensitization (Cornwall and Fain, 1994). However, whether bleaching desensitization requires constitutive stimulation of the visual signaling cascade remains to be determined. Our observation of enhanced activity in the presence of residual retinal oxime, and previous studies of the activity of noncovalent all-*trans* retinal/opsin complexes (Hofmann et al., 1992; Jager et al., 1995) raise the interesting question of the role of all-*trans* retinal in the physiological events associated with large bleaches.

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