Duration and Amplitude of the Light-Induced cGMP Hydrolysis in Vertebrate Photoreceptors Are Regulated by Multiple Phosphorylation of Rhodopsin and by Arrestin Binding

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ABSTRACT: The duration and amplitude of the light-induced cGMP hydrolysis in bovine rod outer segments were investigated using purified rhodopsin in nine different states of phosphorylation in a reconstituted system. Effects of varying amounts of arrestin at all states of rhodopsin phosphorylation were measured. The findings were the following: (1) At low bleaching levels, the activity of phosphodigesterase (PDE) depends strongly on the phosphorylation degree of the light-activated rhodopsin (R*), while at saturating light levels R* of all phosphorylation degrees activates PDE to the same extent. (2) The turnoff time for PDE is markedly shortened if R* is phosphorylated, independent of the number of phosphate groups incorporated into rhodopsin (P/R); i.e., the first phosphate which is bound to R* seems to be responsible for the shortened turnoff time. The lifetime of phosphorylated R* is shown to be dramatically reduced compared to that of unphosphorylated R*, as monitored by the ability of R* to activate PDE. (3) After activation with phosphorylated R*, addition of arrestin caused a further reduction of both the maximum activity and the turnoff time of PDE. Both effects were strongly dependent on (a) the phosphorylation degree of R*, (b) the concentration of arrestin, and (c) the concentration of R*. These results suggest that the light-induced phosphorylation of rhodopsin to different extents and the subsequent binding of arrestin are involved in the light adaptation and in the fine regulation of the light response in vertebrate photoreceptors.

Absorption of light by the visual pigment rhodopsin of vertebrate photoreceptor leads to its conversion into an "active state" (R*), which has been shown to be meta-rhodopsin II (Emeis et al., 1982; Bennett et al., 1982; Kibelbek et al., 1991). R* interacts with a G-protein, called transducin, thereby initiating the exchange of GTP for bound GDP at the α-subunit of transducin (Fung & Stryer, 1980). Binding of GTP leads to the dissociation of the α-subunit of transducin from the βγ-subunits and from R* (Kühn, 1980). The GTP-binding form of the α-subunit activates a phosphodiesterase (PDE) (Fung et al., 1981; Uchida et al., 1981) by inactivating its two inhibitory subunits (Hurley & Stryer, 1982; Deterre et al., 1988; Catty et al., 1992). Since binding and dissociation of transducin take only about a millisecond (Yuong et al., 1984), a single R* can interact sequentially with hundreds of transducin molecules, thereby activating in less than a second hundreds of PDE molecules. The resulting decrease in cGMP concentration causes the closure of cation channels in the plasma membrane, which are kept open by cGMP (Fesenko et al., 1985; Zimmermann et al., 1985; Haynes et al., 1986).

A fast recovery from the light response requires rapid and effective inactivation of PDE. Transducin inactivates itself by an intrinsic GTPase activity of T_α (Fung, 1983; Dratz et al., 1987; Arshavsky et al., 1989). In order to prevent recycling of regenerated transducin, R* has to be effectively inactivated as well.

Inactivation of R* is achieved in two steps: first, R* becomes phosphorylated at several sites by a rhodopsin kinase. Up to nine phosphate groups can be bound to R* (Kühn & Dreyer, 1972; Bownds et al., 1972; McDowell & Kühn, 1977; Wilden & Kühn, 1982), which reduces the ability of R* to activate transducin (Sitaramayya & Liebman, 1983; Miller & Dratz, 1984; Wilden et al., 1986a). In a second step, a cytosolic protein, called arrestin, binds to phosphorylated R*, thereby preventing further interaction with transducin (Wilden et al., 1986a; Bennett & Sitaramayya, 1988; Schleicher et al., 1989).

Recently a protein was discovered, called β-arrestin, which protects the phosphorylated β-adrenergic receptor from binding of G-proteins in a similar way (Lohse et al., 1990). Meanwhile, arrestin-like proteins have been observed in several other systems as well: in various bovine tissues (Scheuring et al., 1990), in turkey erythrocytes (Mirshahi et al., 1989), in the photoreceptor of *Drosophila* (Smith et al., 1990; Matsumoto & Yamada, 1991), in human thyroid (Rapoport et al., 1992), and in yeast cells (Jeansonnet et al., 1991).

As reported earlier (Wilden & Kühn, 1982; Wilden et al., 1986a; Miller et al., 1986), rhodopsin can be phosphorylated to different degrees (1−9P/R). It remained unclear until now if all these differentially phosphorylated rhodopsin species behave differently with respect to binding of arrestin. There is evidence in the literature that an increased level of rhodopsin phosphorylation results in a decreased activation of transducin (Miller et al., 1986; Arshavsky et al., 1987; Bennett & Sitaramayya, 1988). Here I report that the ability of R* to activate the cGMP cascade as well as its binding

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affinity for arrestin strongly depends on the number of phosphate groups bound to R*. These findings along with the light-dependent transport of transducin and arrestin between the inner and outer segment (Philip et al., 1987; Mangini & Pepperberg, 1987; Broekhuyse et al., 1987; Whelan & McGinnis, 1988) reveal a marvelous mechanism of the photoreceptor for fine regulation of the light response and for adaptation to different light levels. Similar mechanisms may be used by other members of the G-protein-coupled receptor family.

MATERIALS AND METHODS

ROS membranes were purified from fresh bovine eyes as described (Wilden & Kühn, 1982). They were stored frozen under argon at −70 °C.

Phosphorylated disk membranes were prepared as described (Kühn et al., 1984). Briefly, ROS were suspended in 100 mM phosphate buffer and illuminated for 3 h at 30 °C in the presence of 3 mM [γ-^{32}P]ATP (New England Nuclear) and 2 mM $MgCl_2$. The resulting [^{32}P]opsin was regenerated by incubating it with a 3−4-fold molar excess of 11-*cis*-retinal (kindly provided by Hoffmann-LaRoche) for 15 h at room temperature in the dark. The regenerated membranes (regeneration yield 100−105%) were washed twice with 100 mM sodium phosphate buffer and twice with 5 mM Hepes, pH 7.2. The final pellet was stored under argon at −70 °C. The average phosphorylation degree of these membranes was usually 5.5−6.5 phosphates incorporated per rhodopsin (P/R). Unphosphorylated control disk membranes were treated in the same way, but in the absence of ATP. After 2 weeks at −80 °C or 3 days at 4 °C, no loss of radioactivity was measured in the phosphorylated preparations.

Preparation of homogeneously phosphorylated rhodopsin species (phosphorylation degrees from 0 to 9 P/R) in detergent solution was performed as described by Wilden and Kühn (1982). Briefly, phosphorylated disk membranes were solubilized with 2% emulphogene in 20 mM phosphate buffer and applied to a column of concanavalin A−Sepharose 4B (Pharmacia). After the column was washed with phosphate buffer, rhodopsin was eluted in 1 mM phosphate buffer containing 2% emulphogene and 200 mM methyl α-D-mannoside (Sigma, grade III).

In order to separate differently phosphorylated rhodopsin species from each other, the eluate of the concanavalin A column was put on top of a cascade of 8−14 small columns filled with epichlorohydrin triethanol−cellulose (ECTEOLA−cellulose, Bio-Rad) and allowed to flow serially through all of the columns. Each of the columns bound as much [^{32}P]-rhodopsin as was allowed by its binding capacity; unbound [^{32}P]rhodopsin was transferred to the next column. The bound [^{32}P]rhodopsin was then eluted from each column with a buffer containing 100 mM sodium phosphate, pH 7.0, 300 mM NaCl, and 2% emulphogene. Highly phosphorylated rhodopsin species were eluted from early columns and lower phosphorylated rhodopsins from later ones. Unphosphorylated rhodopsin was not bound by ECTEOLA−cellulose at all and run through the whole column cascade, as well confirmed by binding experiments with unphosphorylated rhodopsin preparations. Because the binding capacity of the ECTEOLA−cellulose for rhodopsin depends dramatically on the phosphorylation degree of rhodopsin [see Wilden and Kühn (1982)], later columns had to be larger and larger in size than the very small early columns. The binding capacity for rhodopsin with 1 P/R, for example, is only ca. 1.3% of that for rhodopsin with 9 P/R. This excludes significant contaminations of the highly phosphorylated rhodopsin fractions with low phosphorylated rhodopsin. Analytical rechromatography of the fractions of the column cascade shows that they contain two adjacent phosphorylation degrees of rhodopsin, with no measurable contaminations by other phosphorylation degrees. For example, a fraction with 3.7 P/R contained 30% of rhodopsin with 3 P/R and 70% of rhodopsin with 4 P/R. Additionally, the purity of the fractions of the column cascade was controlled by analytical isoelectric focusing (Figure 1). Finally, the emulphogene was exchanged for 2% *n*-octyl glucoside using a concanavalin A column. The eluates from this last column were stored in aliquots at −70 °C until use.

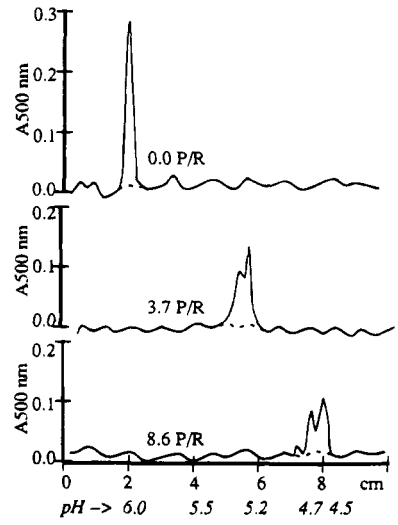

FIGURE 1: Analytical isoelectric focusing of rhodopsin fractions with different phosphorylation degrees isolated by the ECTEOLA−cellulose column cascade. Shown are the absorption scans at 500 nm of dark kept gels before (solid line) and after bleaching (dotted line).

Protein concentration was determined by Bradford's assay using bovine serum albumin as a standard. Rhodopsin concentration was determined from the difference in light absorbance at 500 nm between bleached and unbleached rhodopsin.

Purified Proteins. Transducin and phosphodiesterase (PDE) were purified according to Kühn (1981). Arrestin was purified using its light-dependent binding to phosphorylated rhodopsin as described in Wilden et al. (1986b).

Isoelectric focusing was performed as described by Kühn (1977).

Phosphodiesterase (PDE) activity was monitored through the pH changes caused by cGMP hydrolysis (Yee & Liebman, 1978). Samples (0.7 mL) were incubated in a cuvette thermostatically regulated at 30 °C and equipped with a mechanical stirrer and with a pH electrode (Amagrus). The pH meter was connected to a pen-recorder and a computer. The assay mixture contained 150 mM KCl, 10 mM Hepes, pH 7.9, 1 mM $MgCl_2$, 1 mM dithiothreitol, 4 mM cGMP, 0.5 mM GTP, and purified proteins and other components as indicated. Samples were preincubated in the dark for 5 min. The reaction was normally started by bleaching a calibrated amount of rhodopsin with a photographic flash

(100 μs duration) filtered through a green filter (500 nm) and attenuated with neutral density filters. All PDE assays were performed in complete darkness.

Preparation of Lipid Vesicles. Crude phosphatidylcholine (Sigma, type II-S) was extracted with chloroform, dried under nitrogen, and suspended in 10 mM Hepes (pH 7.9)−150 mM KCl (20 mg/mL). The suspension was sonicated for 4 × 30 s with a Branson B12 sonicator and stored in aliquots at −70 °C until use. This vesicle preparation was used for PDE assays in the "lipid associated" system. For PDE experiments using the "lipid associated" system, 9 μL (≅180 μg) of preformed lipid vesicles was diluted into 681 μL of PDE buffer containing cGMP, GTP, purified transducin (0.45 nmol), and PDE (0.045 nmol). The mixture was incubated for 5 min at 30 °C. Ten microliters (≅1.8 μg) of purified homogeneously phosphorylated rhodopsin in 2% octyl glucoside was added to the prewarmed mixture in complete darkness and allowed to associate with the lipid vesicles for another 5 min. The final concentration of octyl glucoside was ca. 0.03%, well below the critical micelle concentration. The reaction was started with a calibrated flash of light. This procedure was used in all experiments shown in Figures 2−7.

RESULTS

Influence of Different Phosphorylation Degrees on the Ability of Rhodopsin To Activate Phosphodiesterase (PDE). To investigate the ability of chromatographically isolated rhodopsin species of different phosphorylation degrees (P/R), to activate PDE after a flash of light, a system with rhodopsin incorporated into lipid vesicles has turned out to be unsatisfactory for the following reasons:

(1) Using vesicles with rhodopsins phosphorylated to different extents, reassociation of vesicles with purified transducin and PDE became more and more incomplete with increasing P/R, possibly due to the increasing negative charge of the vesicle membranes. Even after a saturating flash of light, highly phosphorylated vesicles activate PDE only to ca. 50−60%, as compared to unphosphorylated vesicles. Moreover, the same difference between highly phosphorylated and unphosphorylated vesicles is seen when PDE is activated in the dark with purified $T_\alpha GTP\gamma S$ (Dizhoor et al., 1985). It will be shown later that the latter two results can be regarded as artifacts due to the properties of these vesicles.

(2) Vesicles with differently phosphorylated rhodopsin species seem to be different in size distribution, as judged by centrifugation experiments. This is highly undesirable, because the light sensitivity of the reconstituted system depends on vesicle size: larger vesicles lead to enhanced light sensitivity as compared to smaller ones.

(3) The fraction of rhodopsin molecules incorporated with the correct orientation in the vesicle membrane may strongly depend on the phosphorylation degree. Unfortunately, this parameter cannot be controlled.

For these reasons, a system was developed in which rhodopsin is not incorporated into lipid vesicles, but associated with the membrane of preformed vesicles. The advantages of this method are:

(1) The number of rhodopsin molecules per vesicle can be made very low, thus preventing an excessive negative charge density of the vesicle surface.

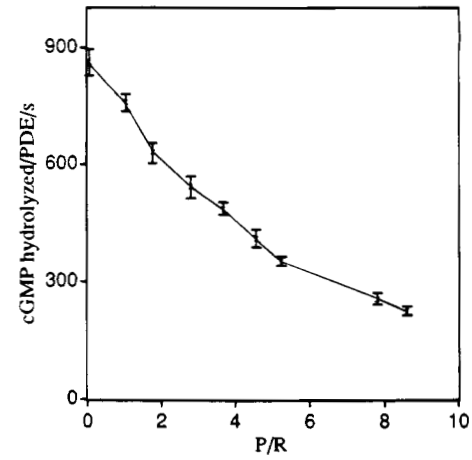

FIGURE 2: Maximum velocity of cGMP hydrolysis by PDE after a dim flash of light, depending on the phosphorylation degree of rhodopsin (P/R). Each sample contained 180 μg of preformed lipid vesicles (see Materials and Methods) and 1.8 μg of homogeneously phosphorylated rhodopsin peripherally associated with the surface of the vesicles, 0.45 nmol of purified transducin, and 0.045 nmol of purifed PDE (which corresponds to 180 μg of rhodopsin in native ROS) in a final volume of 0.7 mL. The reaction was started by a flash of light bleaching 0.18 μg of rhodopsin. The concentration of all components (i.e., transducin, PDE, lipids, and R*) is exactly the same as in a suspension of ROS containing 180 μg of rhodopsin. Only the concentration of unbleached rhodopsin, which does not take place in the reaction, is different. That means the ratio of transducin and PDE to R* is the same as in a ROS suspension after a flash bleaching a fraction of 10^{-3} of the rhodopsin (n = 6).

(2) Since rhodopsin is not incorporated into the vesicles, it cannot be incorporated "inside out".

(3) A single preformed vesicle population can be used for the whole series of experiments with rhodopsin species of all phosphorylation degrees from 0 to 9 P/R. This eliminates an important source of experimental error, namely, the different size distribution of different vesicle preparations. Measurements using this method are highly reproducible; the only disadvantage is the relatively poor light sensitivity, as compared to native disk membranes.

Figure 2 shows the maximum velocity of light-induced cGMP hydrolysis, depending on the light phosphorylation degree of rhodopsin (0−8.6 P/R). The ability of bleached rhodopsin (R*) to activate PDE decreased steadily with increasing ratios of P/R, but even highly phosphorylated R* was still able to activate PDE. A flash, bleaching 0.18 μg of rhodopsin, led with unphosphorylated rhodopsin to ca. 60% PDE maximum activity, as compared to the maximum activity at saturating light levels; with 8−9-fold phosphorylated rhodopsin, however, the same flash led to only 15.6% saturation. Thus, at this intermediate light level, phosphorylation of rhodopsin inhibits PDE activity up to 74%, as shown in Figure 2. Lower light levels led to stronger quenching effects by phosphorylation, even more than 90% at very dim flashes. However, here it became more and more difficult to measure such low activities precisely, so I cannot define an exact limit for the quenching effect of rhodopsin phosphorylation at extremely dim light levels. At saturating light levels, PDE activity corresponded to 1398 ± 31 cGMP PDE^{-1} s^{-1} for all phosphorylation degrees in this "lipid associated" system. To induce this saturated PDE activity, rhodopsin phosphorylated to the highest extent required about 10 times more light than unphosphorylated rhodopsin. That

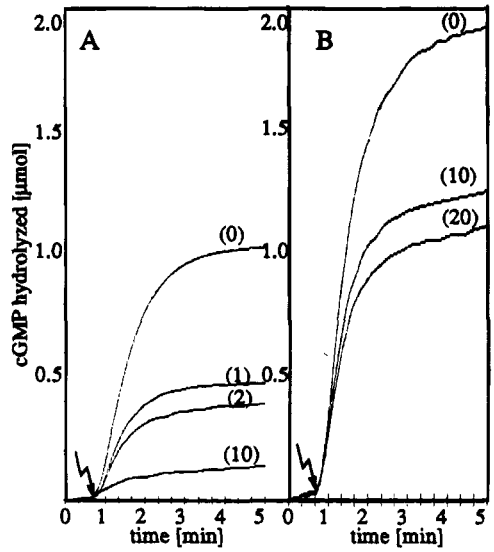

FIGURE 3: PDE activity initiated by R* with a phosphorylation degree of 7.8 P/R (A) or 1.8 P/R (B) in the presence or absence of arrestin. Each number in parentheses denotes the amount (micrograms) of arrestin added to the corresponding sample. The reaction was started by a flash of light bleaching 0.18 μg of rhodopsin. The arrows indicate the time when the flash was delivered. With the exception of arrestin added, the sample composition was the same as in Figure 2 (n = 5).

means at very high light intensities PDE activity became independent of the phosphorylation degree of R*.

*Influence of Arrestin on the Maximum Activity of PDE at Different Phosphorylation Degrees of R**. Arrestin binds to illuminated, phosphorylated rhodopsin already at a phosphorylation degree as low as 1 P/R, thereby partly quenching its ability to activate PDE, but for comparable quenching effects much less arrestin is needed at higher phosphorylation degrees. In Figure 3, the time courses of cGMP hydrolysis are compared in the presence or absence of arrestin at phosphorylation degrees of 7.8 and 1.8 P/R. With 7.8 P/R, as little as 1 μg of arrestin is sufficient to produce a much stronger quenching effect than a 20-fold amount of arrestin at 1.8 P/R. Figure 4 shows a block diagram of the maximum activity of PDE at nine different phosphorylation degrees of rhodopsin and different concentrations of arrestin, normalized to the percent of maximum activity with 0.0 P/R in the absence of arrestin. With increasing P/R, the quenching effect of arrestin on PDE activity becomes continuously more and more pronounced at the same concentration of arrestin; i.e., with increasing P/R, less arrestin is needed to achieve a given quenching effect. As shown in Figure 5, the number of arrestin molecules per bleached rhodopsin necessary for 50% inhibition of maximum PDE activity increased dramatically with decreasing P/R. With only 1.0 phosphate bound to rhodopsin, as many as 400 arrestin molecules per R* were needed to achieve a 50% quenching effect on the peak velocity of cGMP hydrolysis; with 8.6 P/R, for the same effect already 4 molecules of arrestin are sufficient.

This was the situation under in vitro conditions, where all components are highly diluted as compared to their concentrations in the intact ROS, and surely it cannot quantitatively correspond to the situation in vivo. Nevertheless, these results clearly demonstrate that the binding capacity of rhodopsin for arrestin changes with the extent of phosphorylation.

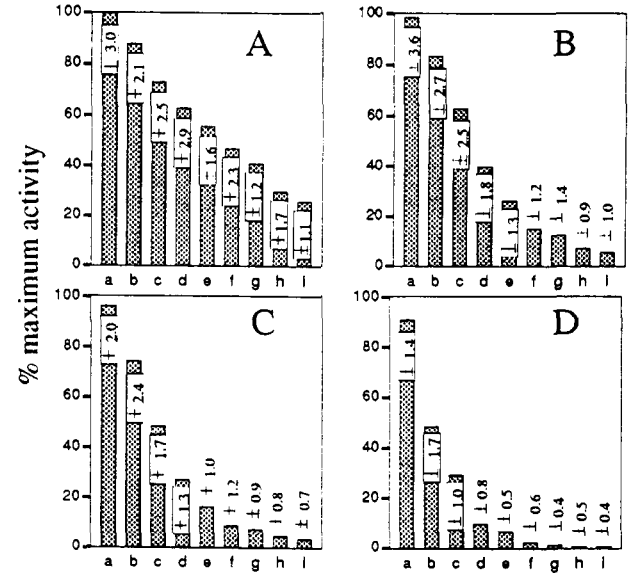

FIGURE 4: Peak velocity of cGMP hydrolysis initiated by bleaching of 0.18 μg of rhodopsin with different numbers of phosphate incorporated, in the presence of different amounts of arrestin and normalized to the percent of peak activity initiated with unphosphorylated rhodopsin in the absence of arrestin (=100%), which corresponds to 840 cGMP PDE^{-1} s^{-1}. Phosphorylation degrees of rhodopsin are (a) 0.0, (b) 1.0, (c) 1.8, (d) 2.8, (e) 3.7, (f) 4.6, (g) 5.2, (h), 7.8, and (i) 8.6 P/R. With the exception of arrestin, the sample composition and initiating flash are the same as in Figures 2 and 3: (A) no arrestin added; (B) 5 μg; (C) 10 μg; (D) 50 μg of arrestin added to each sample (n = 5).

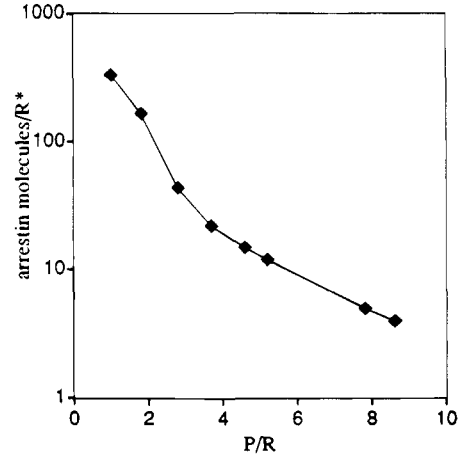

FIGURE 5: Numbers of arrestin molecules per R* necessary for 50% inhibition of peak activity of PDE depending on the phosphorylation degree of rhodopsin. Samples with rhodopsin of every phosphorylation degree were titrated with increasing amounts of arrestin in order to estimate the number of arrestin molecules per bleached rhodopsin which inhibits PDE peak activity by 50% at each phosphorylation degree. The 50% point of each titration curve was plotted against the phosphorylation degree of the corresponding sample (n = 5).

Influence of the Phosphorylation State of Rhodopsin and of Arrestin Binding on the Turnoff Time of PDE in the "Lipid Associated" System. In order to shut off PDE activity after a flash of light, all components of the light-induced enzyme cascade (i.e., R*, transducin, and PDE) must return to their resting state. The PDE turnoff time should depend on (a) the lifetime of R* and (b) the GTPase activity of T_αGTP and the release of PDE inhibitor from T_α. Figure 6A shows the time courses of PDE activity after a flash of light bleaching 0.18 μg of rhodopsin (derivatives of the time

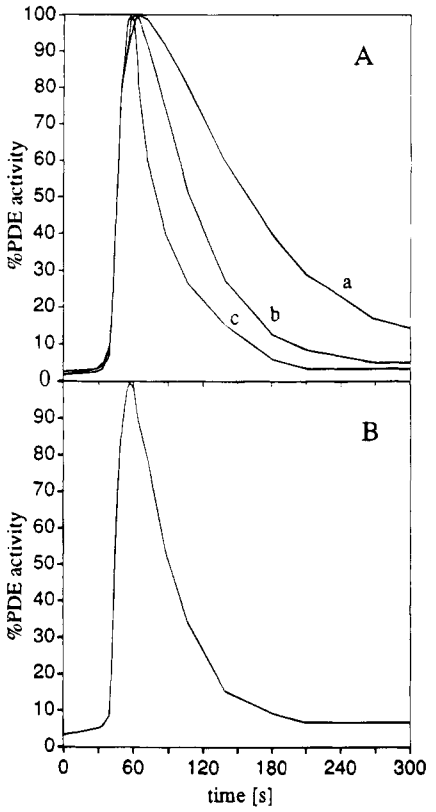

FIGURE 6: (A) Time courses of PDE activity (derivatives of the time courses of cGMP hydrolysis, normalized to 100% peak activity) initiated by bleaching 0.18 μg of (a) unphosphorylated rhodopsin, (b) 1-fold phosphorylated rhodopsin, and (c) 1.8-fold phosphorylated rhodopsin + 253 μg of arrestin (=1200 arrestin molecules/R*). (B) Time course of PDE activity corresponding to a single GTPase cycle of transducin, achieved by a 3.3-fold molar excess of transducin over GTP, and by starting the reaction with a saturating flash of light. The low concentration of GTP prevented recycling of transducin with R* (n = 6).

Table 1: Turnoff Times of the PDE Activities Induced by Illumination of Rhodopsin Species with Different States of Phosphorylation[a]

P/R	PDE turnoff times (1/e)	P/R	PDE turnoff times (1/e)
0.0	145 ± 4.7	4.6	80.0 ± 3.0
1.0	79.4 ± 3.0	5.2	81.5 ± 2.4
1.8	81.4 ± 2.5	7.8	82.0 ± 2.6
2.8	81.1 ± 2.8	8.6	82.3 ± 1.8
3.7	80.8 ± 2.1		

[a] The turnoff times (1/e) were estimated from the PDE activity time courses like those shown in Figure 6A (n = 6 for all phosphorylation degrees).

courses of cGMP hydrolysis, normalized to 100% peak activity) induced by either phosphorylated or unphosphorylated rhodopsin and in the presence or absence of arrestin. Incorporation of 1−9 phosphate groups into rhodopsin clearly shortened the time course of light-induced PDE activity, as compared to the time course of PDE activity initiated by unphosphorylated rhodopsin. A single phosphate group was sufficient for this effect; further binding of up to nine phosphate groups did not further shorten the PDE turnoff time. Bleaching rhodopsin with phosphorylation degrees from 1 to 9 P/R produced identical time courses of PDE activity within the limits of experimental error. Table 1 shows the PDE turnoff times (1/e) for all phosphorylation degrees of R* from 0 to 8.6 P/R. There seems to be one

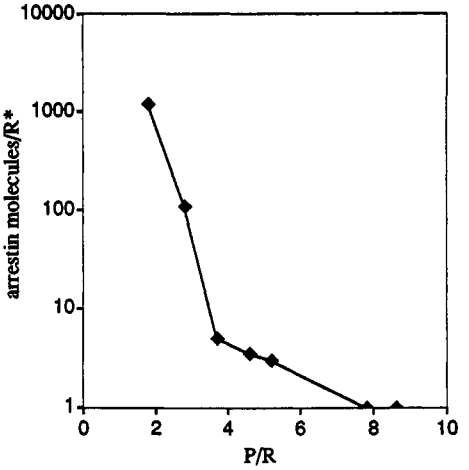

FIGURE 7: Number of arrestin molecules/R* necessary to shorten the PDE turnoff time to the turnoff time corresponding to a single GTPase cycle of transducin, depending on the phosphorylation state of the activating rhodopsin. Activity time courses were taken with different amounts of arrestin added to the sample, and for each phosphorylation degree of rhodopsin, the minimum amount of arrestin was estimated, which was able to make the activity time course identical to that observed when only a single GTPase cycle is allowed to transducin, i.e., that amount of arrestin which after a short association time is able to shield R* completely against further interactions with transducin (n = 5).

particular phosphorylation site on rhodopsin at which phosphorylation is always started and which is involved in controlling the lifetime of the active state of rhodopsin.

Further shortening of the activity time course required the addition of arrestin. In the presence of arrestin, the time course became similar to that corresponding to a single GTPase cycle of transducin in the given system. Figure 6B shows the time course of PDE activity corresponding to a single GTPase cycle, achieved by a GTP/transducin ratio of only 0.3, i.e., only 0.14 nmol of GTP, and activation of the system with a saturating flash of light. Under these conditions, only 30% of the transducin present can be activated by a saturating flash, because of the limiting concentration of GTP, and recycling of transducin by R* is eliminated. The amount of arrestin needed to shorten the PDE turnoff time to that of a single GTPase cycle depended dramatically on the phosphorylation state of rhodopsin (see Figure 7). For 7.8 or 8.6 P/R, a single arrestin molecule per bleached rhodopsin was sufficient, while for 1.8 P/R, as many as 1200 arrestin molecules were necessary per bleached rhodopsin. With 1.0 P/R, not even 5000 arrestin molecules per R* were able to shorten the turnoff time significantly. It was not possible to shorten the PDE activity time course below that of a single GTPase cycle of transducin in the given system, even at a very large excess of arrestin and high phosphorylation degrees of rhodopsin. One would expect this result, if the velocity of GTP hydrolysis is limiting for PDE turnoff.

Time Course of PDE Activity in Native Disk Membranes: Influence of Rhodopsin Phosphorylation and Arrestin. Using native disk membranes, either prephosphorylated or unphosphorylated (see Materials and Methods), the difference in time courses of PDE activity between phosphorylated and unphosphorylated membranes was even more pronounced than in the "lipid associated" system (see Figure 8A). In this experiment, instead of using the same bleaching level for phosphorylated and unphosphorylated disks, I chose

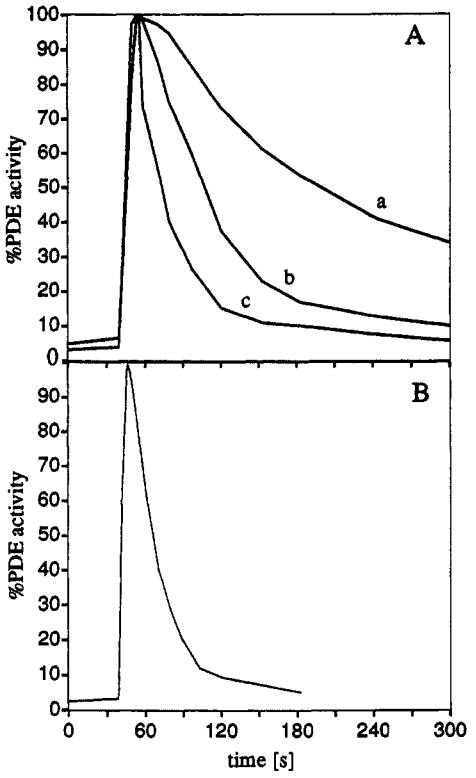

FIGURE 8: (A) Time courses of PDE activity (derivatives of the time courses of cGMP hydrolysis, normalized to 100% peak activity) in a "native disk system". Purified transducin (0.4 nmol) and PDE (0.04 nmol) were recombined with washed disk membranes containing 4 nmol of rhodopsin. (a) Unphosphorylated "control disk membranes"; (b) phosphorylated disk membranes (average phosphorylation degree 6.5 P/R); (c) phosphorylated disk membranes in the presence of 40 μg of arrestin. Each sample contained 500 μM GTP and 4 mM cGMP. The reaction was started in both membrane systems by a ca. half-saturating flash of light, bleaching a fraction of 2.5×10^{-5} of the rhodopsin for unphosphorylated and 1×10^{-4} for phosphorylated rhodopsin. (B) PDE activity time course at conditions allowing just a single GTPase cycle of transducin. Unphosphorylated control disk membranes were recombined with transducin and PDE as in (A), but the sample contained only 0.12 nmol of GTP; i.e., transducin was present in 3.3-fold molar excess over GTP. The reaction was started by a saturating flash of light and stopped by exhaustion of GTP (n = 4).

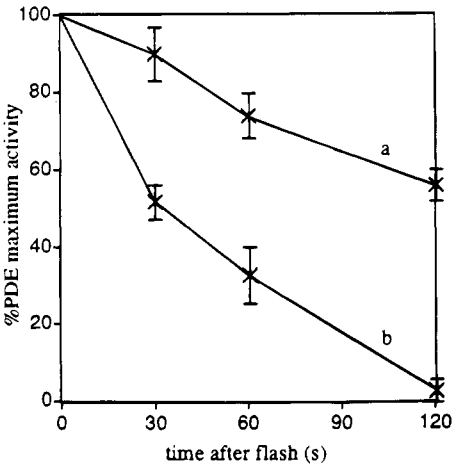

FIGURE 9: Time courses of relaxation of the active states of unphosphorylated (a) and phosphorylated (b) rhodopsin, monitored by measuring the capacity to initiate cGMP hydrolysis at different times after the flash. Washed disk membranes were exposed to a half-saturating flash, bleaching a fraction of 2.5×10^{-5} of the rhodopsin for unphosphorylated and of 1×10^{-4} for phosphorylated disks, in the presence of purified PDE, cGMP, and GTP, but in the absence of transducin. At the indicated times after flash, cGMP hydrolysis was started by addition of 0.4 nmol of transducin. PDE peak activity at each time was estimated and normalized to 100% peak activity at time zero (=flash and addition of transducin simultaneously). 100% peak activity corresponds to 980 cGMP PDE^{-1} s^{-1} for unphosphorylated and to 680 cGMP PDE^{-1} s^{-1} for phosphorylated disk membranes (n = 5).

rather for both membrane types the ca. half-saturating flash, bleaching a fraction of 2.5×10^{-5} of the rhodopsin for unphosphorylated disks and a fraction of 1×10^{-4} for phosphorylated disks. If PDE was activated with phosphorylated R*, it returned much faster to its inhibited state than after activation with unphosphorylated R*. If the same low bleaching level was used for both membrane types, differences in PDE activity time courses were even larger than shown in Figure 8A. This result indicates that phosphorylated R* seems to relax much faster into its inactive state than does unphosphorylated R*. Addition of arrestin to phosphorylated but not to unphosphorylated disks further reduced the PDE activity time course to values corresponding to a single GTPase cycle (compare line c in Figure 8A with Figure 8B). In the "native disk system", the number of arrestin molecules per bleached rhodopsin required for shortening the activity time course to that of a single GTPase cycle and for reduction of the maximum activity was much larger than in the "lipid associated" system. For an average of 6.5 P/R, as many as 475 arrestins/R* were needed for inhibition of maximum activity by 50%, and ca. 2000 arrestins/R* for shortening the activity time course (data not shown). The reason is that a disk membrane preparation with an average phosphorylation degree of 6.5 P/R always contains ca. 12−14% of rhodopsin with low phosphorylation degrees of 0−3 P/R [see Wilden and Kühn (1982)], which require a very high arrestin/R* ratio for both effects, reducing the maximum activity and shortening the turnoff time (see Figures 5 and 7).

Lifetime of the "Active" State of Bleached Phosphorylated and Unphosphorylated Rhodopsin As Monitored by Its Capacity To Activate the Light-Induced Enzyme Cascade after a Dim Flash of Light. In order to investigate the lifetime of the active state of bleached phosphorylated or unphosphorylated rhodopsin, native disk membranes recombined with purified PDE were used. Suspensions of washed disk membranes were bleached with a half-saturating flash (bleaching a fraction of 2.5×10^{-5} of the rhodopsin present for unphosphorylated and a fraction of 1×10^{-4} for phosphorylated disks) in the presence of cGMP, GTP, and PDE, but in the absence of transducin. The reaction was started at different times after the flash by addition of 0.4 nmol of transducin. As can be seen in Figure 9, the capacity to activate the light-induced enzyme cascade (i.e., the "active state" of rhodopsin) decayed much faster in phosphorylated than in unphosphorylated rhodopsin.

SDS−PAGE electrophoresis and silver staining of the washed membrane preparations and of the purified proteins showed no contaminations by arrestin. However, even traces of arrestin, possibly undetected by the silver staining, may perhaps be able to influence the values with the phosphorylated disks, due to the highly phosphorylated rhodopsin species in the mixture. To exclude this possibility, I did the

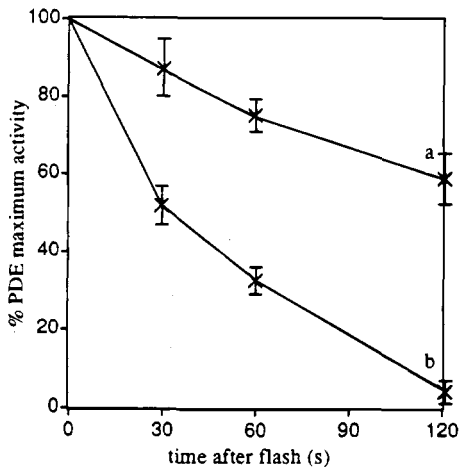

FIGURE 10: Time courses of relaxation of the active states of unphosphorylated rhodopsin (a) and rhodopsin with a defined low phosphorylation degree of 1.8 P/R (b) in a reconstituted vesicle system. Two rhodopsin fractions from the column cascade were reconstituted with isolated ROS specific lipids into vesicles. Samples containing 164 μg of rhodopsin were exposed to a flash of light bleaching a fraction of 5×10^{-4} of the rhodopsin in the presence of purified PDE (0.04 nmol), cGMP, and GTP, but in the absence of transducin. At the indicated times after the flash, cGMP hydrolysis was started by addition of 0.4 nmol of transducin. PDE maximum activity at each time was estimated and normalized as in Figure 9 ($n = 4$).

same experiment as in Figure 9 with purified rhodopsin containing 1.8 P/R and 0 P/R in a reconstituted vesicle system. Two rhodopsin fractions with 0 P/R and with 1.8 P/R were mixed with chloroform-extracted ROS specific lipids. Vesicles were prepared by dialyzing out the detergent (octyl glucoside). This vesicle system is less light-sensitive than the native disk system; a flash bleaching a fraction of 5×10^{-4} led to ca. 68% saturation with 0 P/R and to 51% saturation with 1.8 P/R. Each sample contained 164 μg of rhodopsin. A flash of 5×10^{-4} therefore bleached 0.082 μg of rhodopsin. For a significant quenching effect at 1.8 P/R, at least several hundred arrestin molecules are needed per R* (see Figures 5 and 7); this would correspond to at least 8−16 μg of arrestin. That is more arrestin than a suspension of whole ROS with 164 μg of rhodopsin would contain. Low traces of arrestin, even undetectable by silver stain, would certainly not be able to influence the result in this case. As shown in Figure 10, with the reconstituted vesicle system the results were essentially the same as with the native disk system: the phosphorylated R* again decays much faster than the unphosphorylated R*. This result shows that the accelerated decay is an intrinsic property of the phosphorylated R* and not due to undetected contamination with arrestin.

The "active state" of rhodopsin is supposed to be metarhodopsin II (Emeis et al., 1982; Bennett et al., 1982; Kibelbek et al., 1991). In a recent report (Mitchell et al., 1992), the time course of meta II decay was measured in both phosphorylated and unphosphorylated disk membranes under exactly the same conditions. No differences in meta II kinetics were found between both membrane types. These findings together with the results reported here suggest the idea that the "active state" of bleached rhodopsin may be a spectroscopically indistinguishable substate of meta II rather than being entirely identical with meta II. On the other hand, using native, unphosphorylated ROS membranes, Bennett et al. (1982) and Kibelbek et al. (1991) found similar decay kinetics for meta II and the ability of bleached rhodopsin to catalyze the nucleotide exchange by transducin.

DISCUSSION

At light levels below saturation, the ability of R* to activate the cyclic GMP cascade becomes more and more reduced with increasing numbers of phosphate groups incorporated. This quenching effect of rhodopsin phosphorylation is much more pronounced at low bleaching levels than at higher. So far, my observations are in full agreement with Miller et al. (1986), who used vesicles with incorporated rhodopsin of three different phosphorylation states: 0, 2, and ≥4 P/R. However, even at bleaching levels as high as 10%, they observed an inhibition of 30% with the phosphorylated vesicles. In the assay system with rhodopsin only associated with vesicle membranes, PDE activity initiated by a saturating flash reaches the same value of ca. 1400 cGMP PDE^{-1} s^{-1} at all phosphorylation degrees of R*. The important difference is that with highly phosphorylated rhodopsin much more light is needed for this saturated PDE activity than with unphosphorylated rhodopsin. In the native disk system and also in vesicles with rhodopsin incorporated, I saw the same 30−50% inhibition as Miller et al., even at light levels far above saturation. Furthermore, a 30−40% inhibition was also observed when purified PDE recombined with phosphorylated disk membranes was activated in the dark by addition of purified $T_\alpha GTP\gamma S$ (Dizhoor et al., 1985). The problem seems to be inadequate reassociation of PDE with the strongly negative loaded phosphorylated membranes rather than reduced capacity of phosphorylated rhodopsin to activate PDE. In order to eliminate these sort of artifacts, I preferred an assay system with rhodopsin highly diluted and associated with vesicles rather than incorporated.

Sitaramayya (1986) observed almost total quenching of the light activation of PDE by adding purified rhodopsin kinase to a system containing washed disk membranes, purified transducin, and PDE, in the absence of arrestin. On first sight, this seems to be in contrast with the observations made in our laboratory (Wilden et al., 1986a; and this report), but it is not for the following reasons: (1) Sitaramayya used low bleaching levels (a fraction of 4×10^{-5} of the rhodopsin) and high concentrations of rhodopsin kinase, leading to high phosphate incorporation into rhodopsin within seconds. With low bleaching levels and highly phosphorylated rhodopsin, I saw also a quench of PDE maximum activity up to 90% or, at very dim bleaching levels, even more than 90%. With higher bleaching levels, Sitaramayya did not measure a comparable rapid quench. (2) Once phosphorylated, the lifetime of the active state of rhodopsin is markedly shortened as compared to that of unphosphorylated rhodopsin (see Figures 7A and 8), which leads to a shortened turnoff time for PDE. The interpretation that highly phosphorylated rhodopsin is unable to activate the cyclic GMP cascade is therefore misleading. Addition of arrestin, depending on the phosphorylation state of rhodopsin and of the arrestin concentration, further reduces both the turnoff time and the maximum activity of PDE.

The reduced lifetime of the active state of phosphorylated rhodopsin also explains the findings of Bennett and Sitaramayya (1988) that PDE activity can be turned off by rhodopsin kinase alone and that addition of arrestin accelerates this process.

After activation with phosphorylated rhodopsin, the time course of PDE activity is clearly shortened as compared to the time course with unphosphorylated rhodopsin. This effect is independent of the number of phosphate groups incorporated. My interpretation of this result is that the first phosphate which is bound to rhodopsin alters dramatically the lifetime of R*, whereas binding of the following eight phosphate groups shows no further influence on the decay time of R*. This is partially in agreement with Miller and Dratz (1984), who found also a reduced lifetime of the active state of rhodopsin after phosphorylation. However, in contrast to the experiments reported here, they found a strong dependence on the phosphorylation degree in the range from 1 to 4 P/R. Miller and Dratz prepared disk membranes with 1, 2, 4, and 6 P/R by specific proteolytic elimination of C-terminal phosphorylation sites. Therefore, it remains unclear if 1 P/R means occupation of the same phosphate binding site in the experiments of Miller and Dratz and in mine; if not, this may be the reason for the differences in our results.

Arrestin reduces both the peak activity and the turnoff time of PDE only after activation with phosphorylated R* and in the presence of the GTP-binding form of transducin. When purified PDE is associated with lecithin vesicles and activated with purified $T_\alpha GTP\gamma S$ in the absence of rhodopsin, arrestin shows no effect on PDE activity (Wilden et al., 1986a). Replacing GTP by the nonhydrolyzable analog $GTP\gamma S$ in the presence of phosphorylated R* also eliminates the effect of arrestin on the PDE turnoff time but not on the maximum activity (U. Wilden, unpublished results). These observations together with the result reported here that the quenching effect of arrestin strongly depends on the phosphorylation state of rhodopsin clearly show that arrestin exerts its quenching effect on PDE activity by binding to phosphorylated R* and thereby reduces or (with high P/R and/or high concentrations of arrestin) completely abolishes the capacity of R* to activate transducin and PDE.

The experiments described here clearly demonstrate that rhodopsin phosphorylation in combination with arrestin binding is able to shut off the light-induced enzyme cascade completely. In contrast to my results, Wagner et al. (1988) found that the quenching effect of arrestin requires additional soluble proteins. The reason for this discrepancy seems yet unclear, but from my experiments, I cannot completely exclude the possibility that at low phosphorylation degrees of rhodopsin, where many arrestin molecules per bleached rhodopsin are necessary for complete quenching of the enzyme cascade, additional components may be able to enhance the binding affinity of arrestin for bleached rhodopsin.

At this level, the question of the possible physiological relevance of these findings arises. I will try to address the question according to my present knowledge, which is not really complete at all. The number of cGMP molecules hydrolyzed after a flash of light is dependent on at least five parameters: (1) the number of absorbed photons, i.e., rhodopsin molecules bleached, and (2−5) concentrations of PDE, transducin, rhodopsin kinase, and arrestin. At least three of these five parameters are known to be variables in the rod outer segment: first, of course, the bleaching level; second, the concentration of transducin, because during prolonged light exposure transducin partly moves from the outer segment to the inner segment (Philip et al., 1987; Whelan & McGinnis, 1988); the third component known to be a variable is the concentration of arrestin, which during light adaptation slowly moves into the opposite direction, i.e., from the inner segment to the outer segment (Mangini & Pepperberg, 1987; Broekhuyse et al., 1987; Philip et al., 1987; Whelan & McGinnis, 1988).

In the dark-adapted state, the concentration of transducin is high and that of arrestin low, so that, as an answer to a dim flash, PDE becomes rapidly activated, and, because of fast and high phosphorylation of R* and subsequent arrestin binding, also rapidly inactivated, well before it can reach its maximum activity. A flash of higher intensity also causes rapid activation of PDE, but phosphorylation of R* and arrestin binding take more time, during which more cGMP can be hydrolyzed. At bleaching levels above saturation, phosphorylation of R* may become incomplete, and the arrestin concentration may become critical for PDE turnoff.

After prolonged light exposure, the transducin concentration slowly decreases, and the arrestin concentration increases, whereas rhodopsin kinase (dependent on the intensity and the duration of the background light) may be no longer able to phosphorylate all of the R* up to the maximum level of 9 P/R. In this state, a dim flash causes a slower PDE activation because of the low transducin concentration; but, in spite of the incomplete phosphorylation state of R*, an enhanced arrestin concentration may cause a turnoff as rapid as in the dark-adapted state. The result would be that the same flash initiates the hydrolysis of more cGMP in the dark-adapted state than in the light-adapted state.

A recent report (Mangini et al., 1994) shows that hydroxylamine decreases the movement of arrestin from the inner segment to the outer segment after prolonged light adaptation. Hydroxylamine has also been shown to inhibit the light-dependent phosphorylation of rhodopsin (Pepperberg & Okajima, 1992). These results also suggests a close connection between the phosphorylation state of rhodopsin and the light-induced fixation of arrestin in the outer segment of the rod.

If one assumes that during the process of light adaptation transient axial gradients of R*, transducin, and arrestin concentrations are generated in the outer segment, the situation becomes even more complex, and possibilities for highly differentiated light responses are potentiated. Thus, rhodopsin phosphorylation and arrestin not only can be responsible for a rapid shut-off of the cyclic GMP cascade but also seem to be involved in light adaptation and in the fine regulation of the primary light response.

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REFERENCES

Arshavsky, V. Y., Antoch, M. P., Dizhoor, A. M., Rakhilin, S. V., & Philippov, P. P. (1987) in *The Retinal Proteins* (Ovchinnikov, Yu. A., Ed.) pp 497−504, VNU Science Press, Utrecht, The Netherlands.

Arshavsky, V. Y., Antoch, M. P., Lukjanov, K. A., & Philippov, P. P. (1989) *FEBS Lett. 250*, 353−356.

Bennett, N., & Sitaramayya, A. (1988) *Biochemistry 27*, 1710−1715.

Bennett, N., Michel-Villaz, M., & Kühn, H. (1982) *Eur. J. Biochem. 127*, 97−103.
Bownds, D., Daves, J., Miller, J., & Stahlmann, M. (1972) *Nature (London), New Biol. 237*, 125−127.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Broekhuyse, R. M., Janssen, A. P. M., & Tolhuizen, E. F. J. (1987) *Curr. Eye Res. 6*, 607−610.
Catty, P., Pfister, C., Bruckert, E., & Deterre, P. (1992) *J. Biol. Chem. 267*, 19489−19493.
Deterre, P., Bigay, J., Forquet, F., Robert, M., & Chabre, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2424−2428.
Dizhoor, A. M., Arshavsky, V. Y., Rybin, V. O., & Philippov, P. P. (1985) *Biol. Membr. 2*, 272−276.
Dratz, E. A., Lewis, J. W., Schaechter, L. E., Parker, K. R., & Kliger, D. S. (1987) *Biochem. Biophys. Res. Commun. 146*, 379−386.
Emeis, P., Kühn, H., Reichert, J., & Hofmann, K. P. (1982) *FEBS Lett. 143*, 29−34.
Fesenko, E. E., Kolesnikov, S. S., & Lyubarsky, A. L. (1985) *Nature 313*, 310−313.
Fukada, J., Yoshizawa, T., Saito, T., Ohguro, H., & Akino, T. (1990) *FEBS Lett. 261*, 419−422.
Fung, B. K. K. (1983) *J. Biol. Chem. 258*, 10495−10502.
Fung, B. K. K., & Stryer, L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2500−2504.
Fung, B. K. K., Hurley, J. B., & Stryer, L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 152−156.
Haynes, L. W., Kay, A. R., & Yau, K. W. (1986) *Nature 321*, 66−70.
Hurley, J. B., & Stryer, L. (1982) *J. Biol. Chem. 257*, 11094−11099.
Jeansonnet, N. E., Jazwiski, S. M., & Donoso, L. A. (1991) *J. Biol. Chem. 266*, 1−6.
Kibelbek, J., Mitchell, D. C., Beach, J., & Litman, B. (1991) *Biochemistry 30*, 6761−6768.
Kühn, H. (1977) *FEBS Lett. 3*, 199−203.
Kühn, H. (1980) *Nature 283*, 587−589.
Kühn, H., & Dreyer, W. J. (1972) *FEBS Lett. 20*, 1−6.
Kühn, H., Hall, S. W., & Wilden, U. (1984) *FEBS Lett. 176*, 473−478.
Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., & Lefkowitz, R. J. (1990) *Science 248*, 1547−1550.
Mangini, N. J., & Pepperberg, D. R. (1987) *Jpn. J. Ophthalmol. 31*, 207−217.
Mangini, N. J., Garner, G. L., Okajima, T.-I. L., Donodo, L. A., & Pepperberg, D. R. (1994) *Visual Neurosci. 11*, 561−568.
Matsumoto, M., & Yamada, T. (1991) *Biochem. Biophys. Res. Commun. 177*, 1306−1312.
McDowell, J. H., & Kühn, H. (1977) *Biophys. Struct. Mech. 3*, 199−203.
Miller, J. L., & Dratz, E. A. (1984) *Vision Res. 24*, 1509−1521.
Miller, J. L., Fox, D. A., & Litman, B. J. (1986) *Biochemistry 25*, 4983−4988.
Mirshahi, M., Borgese, F., Razaghi, A., Scheuring, U., Garcia-Romeu, F., Faure, J. P., & Motais, R. (1989) *FEBS Lett. 258*, 240−243.
Mitchell, D. C., Kibelbek, J., & Litman, B. J. (1992) *Biochemistry 31*, 8107−8111.
Pepperberg, D. R., & Okajima, T.-I. L. (1992) *Exp. Eye Res. 54*, 369−376.
Philip, J. N., Chang, W., & Long, K. (1987) *FEBS Lett. 225*, 127−132.
Rapoport, B., Kaufmann, K. D., & Chazenbalk, G. D. (1992) *Mol. Cell. Endocrinol. 84*, R39−R43.
Scheuring, U., Franco, M., Fievet, B., Guizouarn, H., Mirashahi, M., Faure, J. P., & Motais, R. (1990) *FEBS Lett. 276*, 192−196.
Schleicher, A., Kühn, H., & Hofmann, K. P. (1989) *Biochemistry 28*, 1770−1775.
Sitaramayya, A. (1986) *Biochemistry 25*, 5460−5468.
Sitaramayya, A., & Liebman, P. A. (1983) *J. Biol. Chem. 258*, 12106−12109.
Smith, D. P., Shieh, B. H., & Zuker, C. S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1003−1007.
Uchida, S., Wheeler, G. L., Yamazaki, A., & Bitensky, M. W. (1981) *J. Cycl. Nucleotide Res. 7*, 95−104.
Wagner, R., Ryba, N., & Uhl, R. (1988) *FEBS Lett. 235*, 103−108.
Wehner, M., & Kühn, H. (1987) *Adv. Biosci. 62*, 345−351.
Wensel, T., & Stryer, L. (1988) in *Enzyme Dynamics and Regulation* (Chock, P. B., Ed.) pp 102−112, Springer-Verlag, Berlin.
Whelan, J. P., & McGinnis, J. F. (1988) *J. Neurosci. Res. 20*, 263−270.
Wilden, U., & Kühn, H. (1982) *Biochemistry 21*, 3014−3022.
Wilden, U., Hall, S. W., & Kühn, H. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1174−1178.
Wilden, U., Wüst, E., Weyand, I., & Kühn, H. (1986b) *FEBS Lett. 207*, 292−295.
Yamazaki, A., & Bitensky, M. W. (1987) *J. Biol. Chem. 262*, 9324−9331.
Yee, R., & Liebman, P. A. (1978) *J. Biol. Chem. 253*, 8902−8909.
Young, T. M., Chabre, M., & Stryer, L. (1984) *Nature 311*, 659−661.
Zimmermann, A. L., Yamanaka, G., Eckstein, F., Baylor, D. A., & Stryer, L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8813−8817.

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