Phosphorylation of Non-bleached Rhodopsin in Intact Retinas and Living Frogs*

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The photoresponse in retinal photoreceptors begins when a molecule of rhodopsin is excited by a photon of light. Photoexcited rhodopsin activates an enzymatic cascade including the G-protein transducin and cyclic GMP phosphodiesterase. As a result, cytoplasmic cyclic GMP concentration is decreased and the photoresponse is initiated. This process is terminated when rhodopsin is phosphorylated by rhodopsin kinase and subsequently blocked by a protein called arrestin. It has been noted by several investigators that light can cause phosphorylation of not only photoexcited but also non-excited rhodopsin in rod photoreceptors. A goal of this study was to determine how much non-bleached rhodopsin is phosphorylated. To determine how the structural integrity of the photoreceptor influences the extent of non-bleached rhodopsin phosphorylation, we studied the reaction in electropermeabilized rod outer segments, in rod outer segments still attached to isolated retinas and in living frogs. In the first two preparations, we found that the maximum extent of non-bleached rhodopsin phosphorylation was approximately 1% of the total rhodopsin pool. In living frogs, the maximal amount of non-bleached rhodopsin phosphorylation was approximately 1% of the total rhodopsin pool. The highest gain of this reaction has been observed with electropermeabilized frog ROS. Up to 1400 phosphates were incorporated into non-bleached rhodopsin for each rhodopsin bleached by a flash of dim light (Binder et al., 1990). We have called this phenomenon high gain rhodopsin phosphorylation. This gain diminishes at higher light levels when more than one rhodopsin per photoreceptor disc is bleached, and the amount of phosphorylated, non-bleached rhodopsin does not exceed ~1% of the total rhodopsin pool. The gain also substantially diminishes when ROS are fragmented into smaller pieces prior to illumination (Binder et al., 1990). Since the integrity of the ROS structure seems so important for maintaining a high gain of non-bleached rhodopsin phosphorylation, we thought that it is possible that the efficiency of phosphorylation in electropermeabilized ROS might be much lower than in intact photoreceptors. We have now tested this idea by comparing the extent of non-bleached rhodopsin phosphorylation in electropermeabilized ROS and in intact photoreceptors, both on isolated retinas and in the living frog. Frog photoreceptors were used in this study because a large amount of rhodopsin can be harvested from a single animal and also because the highest gain of the non-bleached rhodopsin phosphorylation has been reported for frog (Binder et al., 1990). The levels of the non-bleached rhodopsin phosphorylation in electropermeabilized ROS and isolated retinas were similar and reached ~1% of the total rhodopsin pool. The highest level of the non-bleached rhodopsin phosphorylation, up to ~3% of the total rhodopsin pool, was observed in rod photoreceptors of living animals exposed to relatively dim levels of background light (~20 lx) for a time period of 30 min.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP was obtained from DuPont NEN. N-Octyl-β-D-glucopyranoside was obtained from Calbiochem (San Diego, CA). 11-is-Retinal was kindly supplied by Dr. Rosalie Crouch (Medical University of South Carolina). All other chemicals were obtained from Sigma. Bullfrogs (Rana catesbeiana and Rana grylio) were obtained from Niles Biological (Sacramento, CA).

Preparation of Rhodopsin Samples from Living Animals and Isolated Retinas—In experiments looking at phosphorylation in retinas of living frogs, the whole bullfrog was illuminated from above. The animal was then quickly sacrificed by decapitation and the head plunged into liquid nitrogen (de Azeredo et al., 1981; Barbehenn et al., 1986). The head was placed on dry ice under infrared illumination and the front of the eye
including the lens was removed with an electric drill using a plug bit. The frozen vitreous was removed with a serrated cork borer. The retina was then scraped off the back of the eyecup with a cork borer and a sharpened spatula and immediately placed into a quench solution containing 7.5 mM EDTA, 7.5 mM sodium phosphate, 15 mM sodium fluoride, 2 mM dithiothreitol, pH 7.0, to prevent further phosphorylation/dephosphorylation of rhodopsin.

In other experiments, retinas from bullfrogs were isolated as described previously (Bierna and Bownds, 1985) and maintained in oxygenated Ringer’s solution that contained 105 mM NaCl, 2 mM MgCl2, 1 mM CaCl2, 2 mM KCl, 10 mM HEPES, 5 mM glucose, 5 mM NaHCO3, pH 7.5 (Perlman et al., 1982; Pepperberg and Okajima, 1992). Retinas were illuminated from above with a calibrated, orange light whose intensity was controlled with neutral density filters. We found that under these conditions maximum rhodopsin phosphorylation occurred at approximately 3 min after the flash. Therefore, reactions were stopped at this time by shaking each retina in 1 ml of the quench solution. Light microscopic examination showed that rods obtained in this manner were ruptured.

The suspension obtained from either procedure was centrifuged at 13,000 rpm for 15 min and the pellet resuspended in buffer containing 5 mM EDTA, 5 mM sodium phosphate, 10 mM sodium fluoride, 2 mM dithiothreitol, pH 7.0. When indicated, we regenerates opsin with 11-cis-retinal for 3 h at room temperature. By comparing duplicates that were not regenerated with retinal, we were able to calculate the amount of rhodopsin (both phosphorylated and non-phosphorylated) bleached in each experimental system. After a second centrifugation, the pellet was solubilized in loading buffer containing 5 mM EDTA, 5 mM sodium phosphate, 10 mM sodium fluoride, 2 mM dithiothreitol, 50 mg bovine serum albumin, pH 7.0. This suspension was centrifuged as above and the supernatant loaded onto a Mono-P column and analyzed as described below. Dark controls were performed without illuminating the frog or retina. All manipulations were done under infrared illumination using infrared image converters.

Chromatofocusing Analysis of Non-Bleached Rhodopsin—The separation of phosphorylated and non-phosphorylated rhodopsin was performed by a modification of chromatofocusing technique described by Aton et al. (1984) and Arshavsky et al. (1986). Samples prepared from either the living animal or from intact retinas were run under infrared illumination on a 1 ml Mono-P column attached to the FPLC chromatography system (Pharmacia). To distinguish non-bleached rhodopsin from all other proteins in the samples the effluent from the column was monitored with a flow-through spectrophotometer (Kratos) at 500 nm. All buffers contained 1.5% (w/v) n-octyl-β-D-glucopyranoside. The flow rate was 0.5 ml/min. The column was equilibrated with start buffer containing 25 mM bis-Tris, 5 mM EDTA, pH 7.1. After loading the sample, the column was first washed by the start buffer to elute non-phosphorylated rhodopsin. Once the absorbance at 500 nm returned to baseline (–10 min), a buffer solution containing 10% (v/v) Polybuffer 74 (Pharmacia), 5 mM EDTA, pH 4.0, was run through the column. This generated a pH gradient from pH 7.0 to 4.5. The column was then washed with 4 ml of 0.5 M sodium sulfate. Fractions containing material absorbing at 500 nm were collected and the amount of non-bleached rhodopsin was measured by difference spectroscopy (Bownds et al., 1971). We typically recovered greater than 89% of the 8–10 nmol of rhodopsin loaded. The sensitivity of this technique allowed us to detect phosphorylation in as little as 0.1% of the total rhodopsin loaded.

In separate experiments to determine the recovery of phosphorylated rhodopsin from the Mono-P column, purified frog ROS were disrupted by freezing in liquid nitrogen and then bleached and phosphorylated for 30 min in a pseudotracellular medium containing 95 mM potassium isethionate, 15 mM sodium isethionate, 2 mM MgCl2, and 15 mM HEPES, pH 7.5, in the presence of 0.5 mM [γ-32P]ATP (200 μCi total). Bleached rhodopsin was regenerated with 11-cis-retinal and the membranes were washed twice with pseudotracellular medium. The pellet was then solubilized and applied on the Mono-P column as described above. The radioactivity of each fraction was determined by scintillation counting. We recovered over 95% of the [32P]radioactivity applied to the column. Fractions that contained radioactivity were precipitated with 30% trichloroacetic acid and subjected to SDS-polyacrylamide gel electrophoresis. The gel was stained, the rhodopsin band excised, and radioactivity determined by scintillation counting. We typically recovered greater than 90% of the [32P]-labeled rhodopsin in a single peak in the sodium sulfate wash. This rhodopsin peak only appeared when the sample was illuminated in the presence of added ATP.

RESULTS

Chromatofocusing Is a Powerful Technique for Quantitative Studies of Rhodopsin Phosphorylation—The main goal of our study was to determine the extent of non-bleached rhodopsin phosphorylation. We used chromatofocusing to separate phosphorylated and non-phosphorylated forms of rhodopsin based on the difference in their isoelectric points. This technique has several advantages over other methods typically employed to measure rhodopsin phosphorylation. Usually, retinas or animals have been supplied with [32P]P, to radiolabel ATP synthesized in the photoreceptors. After illumination the photoreceptors were subjected to SDS-polyacrylamide gel electrophoresis and radioactivity was determined in the rhodopsin band. This was the approach used by Kühn (1974) in the first study to show that in living frogs rhodopsin becomes phosphorylated in light and then dephosphorylates in the dark. A major problem with this method is that it is difficult to determine the specific radioactivity of ATP in ROS at the time of phosphorylation. One solution to this problem was presented in a study by Ohguro et al. (1995) who selectively cleaved the C terminus of rhodopsin containing the phosphorylation sites and then determined its phosphorylation level by mass spectroscopic analysis. Unfortunately, this technique could not be used in our study since it does not distinguish between phosphorylation of bleached and non-bleached rhodopsin.

Separation of non-phosphorylated and phosphorylated rhodopsin on a pH gradient was originally described by Kühn and McDowell (1977) who separated non-phosphorylated bovine rhodopsin from its phosphorylated forms by isoelectric focusing. This method was later used by Fong et al. (1985) to study frog rhodopsin. Aton et al. (1984) reported preparative isolation of bovine non-phosphorylated rhodopsin and its six phosphorylated forms by chromatofocusing in the presence of the non-ionic detergent n-octyl-β-D-glucopyranoside. A fast protein liquid chromatography modification of this method was described by Arshavsky et al. (1986). One advantage of this method over isoelectric focusing is that when rhodopsin samples are separated in the dark, it is easy to determine the amount of phosphorylated, non-bleached rhodopsin by a difference in the absorbance at 500 nm.

We were faced with a methodological problem while adopting the chromatofocusing technique for frog rhodopsin. Phosphorylated forms of frog rhodopsin bind to the Mono-P column more tightly than that of bovine rhodopsin. They are not eluted from the column above pH 4 which is the lowest pH limit of the method. However, we found that they can be eluted as a mixture of rhodopsin molecules containing various numbers of incorporated phosphates by a step of 0.5 M sodium sulfate at pH 4–4.5. Complete recovery of phosphorylated rhodopsin from the column required the presence of 5 mM EDTA in all chromatography buffers. A typical chromatography profile is shown in Fig. 1. A mixture of non-phosphorylated (~99%) and phosphorylated (~1%) rhodopsin was loaded on the Mono-P column at pH 7.1. Then non-phosphorylated rhodopsin was eluted during the wash of the column, the pH gradient was applied, and finally, phosphorylated rhodopsin was eluted by sodium sulfate. We quantified the amount of rhodopsin in each fraction by differential spectroscopy at 500 nm before and after bleaching the sample.

Effect of ROS Structure on the Phosphorylation of Non-bleached Rhodopsin—Our previous study with frog ROS (Bind-er et al., 1990) indicated that the gain of the non-bleached rhodopsin phosphorylation is influenced by the structural integrity of ROS preparation. Indeed, the bleaching of a single rhodopsin in electroporapermeabilized ROS causes incorporation of up to 1,400 phosphates into non-bleached rhodopsin, while in
disrupted ROS the gain was less than 50. This led us to the belief that the high gain rhodopsin phosphorylation in intact photoreceptors might be even more pronounced than in electropermeabilized ROS. In order to check this hypothesis we measured the amount of non-bleached rhodopsin phosphorylation in ROS still attached to isolated frog retinas. Phosphorylation of total rhodopsin has been measured previously in isolated intact frog retinas (Miller and Paulsen, 1975; Kühn and Bader, 1976), but the amount of non-bleached rhodopsin phosphorylation was never been examined. Fig. 1 shows the chromatography profile obtained in an experiment where non-bleached rhodopsin phosphorylation was determined after bleaching \(9 \times 10^7\) of the \(3 \times 10^9\) rhodopsins in each ROS. Approximately 1.2% of non-bleached rhodopsin became phosphorylated in response to this flash. The dependence of non-bleached rhodopsin phosphorylation on the intensity of the test flash is presented in Fig. 2A. The phosphorylation level remains under the detection level of the method if less than 1% of the rhodopsin pool is bleached. Phosphorylation of non-bleached rhodopsin reaches a maximum when \(\sim 3\%\) of the rhodopsin pool is bleached and then diminishes with bleaching levels higher than this. Under these conditions, the amount of non-bleached rhodopsin phosphorylation was 1.2 ± 0.7% (mean ± S.D., \(n = 4\)) of the total rhodopsin pool. In contrast, the levels of total rhodopsin phosphorylation measured under identical conditions (but after regeneration of bleached rhodopsin with 11-cis-retinal) increase consistently with the increase of light intensity (Fig. 2B). These data are similar to those obtained with electropermeabilized ROS (Binder et al., 1990), suggesting that the structural integrity of the electropermeabilized ROS preparation is sufficient for a complete manifestation of the high gain rhodopsin phosphorylation phenomenon.

Phosphorylation of Non-bleached Rhodopsin in Living Frogs—To determine the amounts of phosphorylated, non-bleached rhodopsin in living frogs, the animals were exposed to various conditions of duration and intensity of illumination. At the end of the light exposure animals were decapitated and their heads were immediately frozen in liquid nitrogen. The retinas were then scraped from the frozen eye into the “quenching solution” and the amount of phosphorylated non-bleached rhodopsin in these preparations was determined by chromatofocusing. In some experiments we also determined the total amount of phosphorylated rhodopsin (bleached and non-bleached) by regenerating bleached rhodopsin with 11-cis-retinal prior to chromatofocusing. The extent of rhodopsin bleaching in these experiments was calculated from the difference in the samples absorbance at 500 nm before and after regeneration. We found that under these conditions, rhodopsin phosphorylation reached a maximum after 30 min of light exposure and then either stayed constant or slowly declined over the following 2 h (data not shown).

Fig. 3 shows the effect of light intensity on the phosphorylation of rhodopsin in the retinas of living frogs. Phosphorylation of non-bleached rhodopsin showed a maximum value of 2.1 ± 1.4% (mean ± S.D., \(n = 3\)) at a light intensity of 20 lx (Fig. 3A). Phosphorylation of total rhodopsin (Fig. 3B) showed a maximum of \(\sim 6\%\) in the light intensity range of 20–100 lx (5.6 ± 0.3%, mean ± S.D., \(n = 3\)). Phosphorylation of both non-bleached and total rhodopsin decreased at brighter light...
Phosphorylation of Non-bleached Rhodopsin in Living Frogs

Figure 3. Phosphorylation of non-bleached rhodopsin in living frogs. Living frogs were illuminated from above for 30 min at the indicated light intensities. They were then sacrificed and the amounts of phosphorylated and non-phosphorylated rhodopsin were determined as described under "Experimental Procedures." Panel A shows the amounts of phosphorylated non-bleached rhodopsin measured in the absence of regeneration. Panel B shows the amounts of total phosphorylated rhodopsin determined after regeneration of bleached rhodopsin in the samples by 11-cis-retinal. Each symbol represents a separate animal. The lines connect the means of all points obtained at each light intensity.

However, even in this case the total amount of non-bleached phosphorylated rhodopsin did not exceed 3% of the total rhodopsin pool in any single experiment.

DISCUSSION

Phosphorylation of unbleached rhodopsin in rod photoreceptors remains one of the least understood reactions in phototransduction. Even though the first indication for this reaction was obtained in one of the original papers describing the phenomenon of light-dependent rhodopsin phosphorylation (Bownds et al., 1972), its role in phototransduction remains unclear. We developed a chromatic focusing technique to further study this issue. The data presented indicate that the phosphorylation of non-bleached rhodopsin in intact photoreceptors is virtually the same as in electropermeabilized ROS. In isolated retinas this reaction reaches its maximum when ~3% of the rhodopsin pool is bleached and declines with bleaching levels above this (Fig. 2A). This reduction at higher intensities of the test flash might be interpreted in the following way: if the bleached rhodopsin serves as a preferred substrate for the kinase (or kinases) responsible for the high gain phosphorylation, then the appearance of large amounts of photoexcited rhodopsin at higher light levels would efficiently compete with the non-bleached rhodopsin for interactions with the kinase (Dean and Akhtar, 1993). This explanation is consistent with the observation that phosphorylation of bleached rhodopsin at higher light levels is substantially increased (Fig. 2B).

The most important observation in this study is that phosphorylation of non-bleached rhodopsin occurs in rod photoreceptors in living animals. Interestingly, the levels of both bleached and non-bleached rhodopsin phosphorylation in living animals reached a maximum at relatively moderate light intensities (~20 lx) and then surprisingly diminished at higher light intensities. Two potential sources of this decrease in phosphorylation might be a shielding of ROS from light by the migration of pigment epithelium and by constriction of the iris.

What Is the Functional Role of Non-bleached Rhodopsin Phosphorylation?—The most commonly discussed idea concerning the role of non-bleached rhodopsin phosphorylation is that it plays a role in photoreceptor adaptation to high levels of background illumination (see Bownds and Arshavsky (1995), for a review). If a substantial portion of non-bleached rhodopsin was to become phosphorylated during background illumination, its excitation should result in photoreponses with lowered amplitude and shorter duration characteristic of light adapted photoreceptors. The amplitude reduction would result from the reduced ability of phosphorylated rhodopsin to activate transducin, while the shorter duration of the response would result from faster binding of arrestin to pre-phosphorylated rhodopsin. The data reported here do not support this hypothesis. We have found that under optimal conditions of illumination not more 3% of the total unbleached rhodopsin pool in the rod photoreceptor becomes phosphorylated. Therefore, the probability that a photon would excite a phosphorylated rhodopsin is very low. However, our data obtained with rod photoreceptors do not exclude the possibility that the mechanism described above might be present in cones. Cones are known to adapt to higher levels of background light than rods, so they might use a different array of adaptation mechanisms from rods.

One alternative hypothesis is that phosphorylation of a fraction of rhodopsin in the photoreceptor disc membrane causes changes in the membrane physical properties that modify the interaction of certain membrane-associated proteins. A hint for such a mechanism was obtained by Dizhoor et al. (1985) who studied cyclic GMP phosphodiesterase activation by transducin bound to a non-hydrolyzable analog of GTP. In agreement with
other reports (Fung and Nash, 1983; Tyminski and O’Brien, 1984; Malinski and Wensel, 1992) they found that membranes are required for an effective phosphodiesterase activation. They also found that photoreceptor membranes (either dark adapted or bleached) containing phosphorylated rhodopsin support phosphodiesterase activation less efficiently than membranes containing non-phosphorylated rhodopsin.

How Can Non-bleached Rhodopsin Become Phosphorylated?—The mechanism of unbleached rhodopsin phosphorylation remains to be determined. Recent studies have shown that upon binding to bleached rhodopsin, rhodopsin kinase becomes able to phosphorylate a rhodopsin C-terminal peptide corresponding to the major phosphorylation site (Fowles et al., 1988; Palczewski et al., 1991). Perhaps rhodopsin kinase also phosphorylates neighboring unbleached rhodopsin molecules. An alternative possibility is that another kinase acts on rhodopsin.

Newton and Williams (1991, 1993) showed that rhodopsin is the major substrate for protein kinase C in ROS and suggested that this kinase may phosphorylate both bleached and non-bleached rhodopsin in ROS. A good way to distinguish between these two kinases is to study the effects of calcium on high gain rhodopsin phosphorylation. Calcium is shown to stimulate the activity of protein kinase C in ROS (Greene et al., 1995) and suppress rhodopsin phosphorylation through the inhibitory action of recoverin on rhodopsin kinase (Klenchin et al., 1995; Chen et al., 1995). Our recent observations indicate that the high gain reaction is several fold more active at 10 nM than at micromolar calcium levels which favors the role of rhodopsin kinase. This is in agreement with Chen et al. (1995) who have shown that purified rhodopsin kinase reconstituted with urea-treated bovine ROS membranes is able to catalyze high gain rhodopsin phosphorylation and that this reaction is sensitive to recoverin. Nevertheless, we do not exclude the possibility that both kinases are working at the same time.

Another possible mechanism leading to the accumulation of non-bleached phosphorylated rhodopsin in photoreceptors is an inhibition of the dephosphorylation reaction without blocking rhodopsin regeneration by 11-cis-retinal. This possibility is discussed by Biernbaum et al. (1991) who demonstrated that a dim background illumination prevents rhodopsin dephosphorylation in intact frog rods, but not in electroporameabilized ROS. In our study this option could only be realized in experiments with living animals since the endogenous content of 11-cis-retinal in isolated frog retinas is insufficient for regeneration of more than ~1% of the rhodopsin pool (Baumann, 1970; Perlman et al., 1982; Coozaza and Ostrey, 1987). Our results are in general agreement with this hypothesis: the level of non-bleached rhodopsin phosphorylation under conditions permissive for regeneration of rhodopsin (living animals) is about 2-fold higher than under conditions where the opportunity for rhodopsin regeneration is limited (isolated retinas).

A goal of future experiments is to elucidate the molecular mechanism that is responsible for phosphorylation of non-bleached rhodopsin and to determine whether this reaction plays a physiological role in vertebrate photoreceptors.

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