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Amplification of Phosphodiesterase Activation Is Greatly Reduced by Rhodopsin Phosphorylation†

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ABSTRACT: In the vertebrate rod outer segment (ROS), the light-dependent activation of a GTP-binding protein (G-protein) and phosphodiesterase (PDE) is quenched by a process that requires ATP [Liebman, P. A., & Pugh, E. N. (1979) *Vision Res. 19*, 375–380]. The ATP-dependent quenching mechanism apparently requires the phosphorylation of photoactivated rhodopsin (Rho*); however, a 48-kilodalton protein (48K protein) has also been proposed to participate in the inactivation process. Purified species of phosphorylated rhodopsin containing 0, 2, or ≥4 (high) phosphates per rhodopsin (PO_4/Rho) were reconstituted into phosphatidylcholine (PC) vesicles and reassociated with a hypotonic extract from isotonically washed disk membranes that were depleted of 48K protein; PDE activation, in response to bleaching from 0.01% to 15% of the rhodopsin present, was measured. PDE activity was reduced by at least 30% at high fractional rhodopsin bleaches and by greater than 80% at low fractional rhodopsin bleaches in high PO_4/Rho samples when compared to the activity measured in 0 PO_4/Rho controls. A phosphorylation level of 2 PO_4/Rho produced PDE activities that were intermediate between 0 PO_4/Rho and high PO_4/Rho samples at low bleaches, but were identical with the 0 PO_4/Rho samples at high rhodopsin bleaches. Rhodopsin phosphorylation is thus capable of producing a graded inhibition of light-stimulated PDE activation over a limited range of (near physiological) bleach levels. This effect becomes less pronounced as the bleach levels approach those that saturate PDE activation. These results are consistent with increasing levels of phosphorylation, producing a reduction of the binding affinity of G-protein for Rho*.

The outer segment of the vertebrate rod cell contains a number of enzymes that are activated in a light-dependent manner. Each of these enzymatic activities are temporally linked to the photobleaching of the visual pigment rhodopsin. An active form of bleached rhodopsin, Rho*, catalyzes the exchange of GTP for previously bound GDP on a GTP-binding protein (G-protein)[1] (Fung & Stryer, 1980). The G-protein, which posesses a slow intrinsic, GTPase activity (Godchaux & Zimmerman, 1979; Kuhn, 1980; Liebman & Pugh, 1981), is then capable of activating a cGMP-specific phosphodiesterase (PDE); this latter function is lost when the bound GTP is hydrolyzed to GDP.

Bleached rhodopsin is also a substrate for phosphorylation by rhodopsin kinase (Kuhn, 1978; Shichi & Somers, 1978; Liebman & Pugh, 1979; Wilden & Kuhn, 1982; Kuhn & Wilden, 1982; Aton et al., 1984; Sitaramayya, 1986). A considerable body of evidence supports Liebman's hypothesis that the phosphorylation of rhodopsin is the key regulatory step in a rapid, ATP-dependent inactivation of Rho* (Liebman & Pugh, 1979, 1981; Sitaramayya & Liebman, 1983a,b; Aton & Litman, 1984; Miller & Dratz, 1984). A recent report by Sitaramayya (1986) suggests that only 1–2 PO_4/Rho are required for the rapid, ATP-dependent inactivation of Rho*. Other reports, however, suggest that rhodopsin phosphorylation alone is not sufficient for the rapid inactivation of Rho*. Kuhn and co-workers (Kuhn et al., 1984; Pfister et al., 1985) have reported that the G-protein binds to both "phosphorylated Rho*" and unphosphorylated Rho*. In addition, they report that an endogenous 48-kilodalton protein (48K protein) binds

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; cGMP, guanosine cyclic 3′,5′-phosphate; ROS, rod outer segment(s); Rho, rhodopsin; PDE, phosphodiesterase; G-protein, also called GTP-binding protein, GTPase, and transducin; 48K protein, 48-kilodalton protein; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; OG, octyl β-D-glucoside; con A, concanavalin A–Sepharose; KIU, Kallikrein inhibitor unit; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PO_4/Rho, phosphates per rhodopsin; PC, phosphatidylcholine.

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to "phosphorylated Rho*" whereas it apparently does not bind to unphosphorylated Rho* (Kuhn et al., 1984). A recent report by Wilden et al. (1986) indicates that rhodopsin phosphorylation is required for the 48K protein dependent quenching of PDE activation. These experiments, utilizing phosphorylated and regenerated disk membranes, required as much as a 10000-fold excess of 48K protein over Rho* in order to observe the quenching of PDE activation. Kuhn has proposed that the ATP-dependent shutoff of PDE activation results from competition between the 48K protein and the G-protein for binding sites on "phosphorylated Rho*". Zuckerman et al. (1984, 1985) also report that the 48K protein participates in the rapid inactivation of Rho*. These authors propose, however, that the 48K protein is first activated by Rho* or "phosphorylated Rho*" and then quenches cGMP hydrolysis by direct interaction with the G-protein or PDE rather than by competition with G-protein for binding sites on "phosphorylated Rho*" (Zuckerman et al., 1985; Zuckerman & Cheasty, 1986).

The above experiments were performed on preparations of phosphorylated ROS fragments or disk membranes that contained heterogeneous populations of phosphorylated rhodopsin as well as unphosphorylated rhodopsin. For example, disk membrane preparations containing an average of six or more phosphates per rhodopsin will also contain a small amount (ca. <5%) of unphosphorylated rhodopsin (Wilden & Kuhn, 1982; Aton et al., 1984). The intrinsic amplification of the PDE activation cascade (Fung & Stryer, 1980) could allow a small amount of unphosphorylated rhodopsin present in preparations of high average phosphate per rhodopsin ratios to contribute significantly to the overall PDE activity by overwhelming the low levels of PDE activation produced from much larger populations of phosphorylated rhodopsin. In order to unambiguously determine the level of PDE activity produced by the bleaching of phosphorylated rhodopsin, the following experiments have been performed. Phosphorylated rhodopsin species containing 2 or ≥ 4 PO_4/Rho were purified and reconstituted into phosphatidylcholine (PC) vesicles. These reconstituted vesicles were then combined with 48K protein depleted enzyme extracts, containing PDE and G-protein, and only a trace amount of 48K protein. We then compared the ability of rhodopsins containing 0, 2, and ≥ 4 PO_4/Rho to support PDE activation.

Materials and Methods

All preparations were performed under dim, far-red light (Kodak Wratten no. 2) at 4 °C unless otherwise indicated. All buffers were thoroughly bubbled with argon before use.

Preparation and Purification of Phosphorylated Species of Rhodopsin. ROS were isolated from frozen retinas according to the procedure of McDowell and Kuhn (1977) with the following modifications. The isolation buffer contained 100 Kallikrein inhibitor units (KIU)/mL of the protease inhibitor Trasylol (Mobay Pharmaceuticals), and 50 μM diethylenetriaminepentaacetic acid (DTPA) was substituted for ethylenediaminetetraacetic acid (EDTA). Phosphorylation was carried out as described by Wilden and Kuhn (1982) utilizing unlabeled ATP. A 20-min incubation period was sufficient to achieve significant yields of species containing 2, 4, or ≥ 6 PO_4/Rho. The reaction was quenched by adding EDTA to a final concentration of 20 mM. Rhodopsin was regenerated after phosphorylation according to a procedure described by Aton and Litman (1984).

Rhodopsin from phosphorylated, regenerated membranes was purified by affinity chromatography on concanavalin A–Sepharose (Con A) as described by Litman (1982), except that the buffer employed contained 50 mM Tris–acetate (pH 7.0), 62.5 μM $MgCl_2$, 50 μM DTPA, 30 mM octyl β-D-glucoside (OG), and 2 mM DTT. Subsequent chromatofocusing was employed to purify the various phosphorylated species of rhodopsin (Aton et al., 1984). Con A purified rhodopsin was prepared for chromatofocusing by concentrating the sample to approximately 4 mg/mL with an Amicon PM10 membrane, diluting the rhodopsin to 0.5 mg/mL with 25 mM histidine (pH 6.4) in 30 mM OG, and reconcentrating to 4 mg/mL rhodopsin.

The peak column fractions for each species were pooled, the pH of the pooled fractions was adjusted to 7.0, and the samples were concentrated with an Amicon PM10 membrane to approximately 2 mg/mL rhodopsin. Because the chromatofocusing elution buffer contains ampholytes that interfere with isoelectric focusing and vesicle reconstitution procedures, each peak species was further purified by affinity chromatography on Con A as described above. The resulting Con A purified rhodopsin was then concentrated to approximately 3 mg/mL rhodopsin with an Amicon PM10 membrane.

Vesicle Reconstitution Procedure. The isolated phosphorylated species of rhodopsin were reconstituted into egg PC vesicles by the dilution procedure described by Jackson and Litman (1985). The initial phospholipid/protein ratio was 100/1 in all cases. The buffer used for reconstitution of unphosphorylated rhodopsin was 50 mM Tris–acetate (pH 7.0), 62.5 μM $MgCl_2$, 50 μM DTPA, and 2 mM DTT. Phosphorylated samples required additional salts (300 mM KCl, 0.5 mM $MgCl_2$) and metal ion chelator (0.1 mM DTPA) in their buffer to ensure the formation of unilamellar vesicles and to maintain sample stability. The final dialysis step was against a hypotonic buffer containing 10 mM Tris (pH 8.0), 2 mM DTT, and 100 KIU/mL Trasylol. The reconstituted samples were concentrated to approximately 3 mg/mL with an Amicon PM10 membrane, and the final phospholipid/protein ratios were obtained as described previously (Jackson & Litman, 1985).

Isoelectric Focusing of Proteins. Rhodopsin was focused in the dark by using 0.5-mm slab gels that contained 5% (w/v) acrylamide, 0.17% (w/v) *N,N'*-methylenebis(acrylamide), 1.25% (w/v) OG, and 2.7% (v/v) ampholine. The pH gradient from pH 6.5 to pH 3.8 was formed by mixing LKB ampholines, pH 4–6.5 and 3.5–5, in a ratio of 4/1, respectively. The electrode solutions were 0.5 M NaOH and 0.5 M acetic acid. Samples for electrofocusing were prepared by diluting 100 μg of rhodopsin to 2 mL in a Centricon 10 microconcentrator with 5 mM sodium phosphate buffer (pH 7.0) containing 100 mM OG. The sample was concentrated to 100 μL by centrifugation for 4–5 h at 5000 rpm in a Sorvall SS-34 rotor. An aliquot of 15–20 μg of rhodopsin was loaded on a gel and isoelectrically focused for 2 h at 5 °C and 25-W constant power. Before staining, the gel was placed in an aqueous solution of 20% trichloroacetic acid and 15% methanol for 1 h to remove the ampholines and to fix the proteins in position. The gel was then washed for 5 min in destaining solution, which contained 25% ethanol and 8% acetic acid in water. The staining solution consisted of 0.12% (w/v) Coomassie Brilliant Blue R250 in destaining solution. The gel was stained for 10 min in staining solution that had been preheated to 60 °C. Destaining took place until the background was clear.

Preparation of Hypotonic Enzyme Extract. ROS were prepared according to Miller and Dratz (1984) in an isotonic buffer (10 mM Tris, pH 8.0, 60 mM KCl, 30 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.05 mM DTPA, and 100 KIU/mL Trasylol). The disk membrane pellet was homogenized be-

tween each of four washes in isotonic buffer to remove soluble proteins, including the 48K protein. The resulting disk membrane pellet was then resuspended and homogenized in hypotonic buffer (5 mM Tris, pH 8.0, 2 mM EDTA, and 1 mM DTT) at 4 °C. The PDE and G-protein were collected in the supernatants from two washes in hypotonic buffer. The hypotonic extract was then concentrated in an Amicon ultrafiltration cell with a PM10 membrane. The protein content of the hypotonic extract was characterized by SDS–PAGE in a Laemmli-type gel system (Laemmli, 1970) using a 12% acrylamide gel with a 30/1.1 acrylamide/bis(acrylamide) ratio according to Farrer (1980).

Quantitation of 48K Protein in the Hypotonic Protein Extract. Aliquots of protein extract and protein extract "spiked" with incrementally increasing amounts of purified 48K protein were subjected to a radioiodination reaction according to Fraker and Speck (1978). Iodogen was purchased from Pierce and ^{125}I from Amersham. The labeled proteins were resolved by SDS–PAGE, the 48K protein band was excised, and the gel slice was then counted. The *y*-axis intercept of a plot of cpm (^{125}I) vs. the amount of added 48K protein corresponded to the ^{125}I incorporated into the 48K protein that was initially present in the enzyme extract. This quantity allows a direct determination of the preexisting concentration of 48K protein in the hypotonic enzyme extract.

Reassociation of Protein Extract with Reconstituted Vesicles. Rhodopsin-containing reconstituted vesicles were combined with protein extract under hypotonic conditions and dialyzed against isotonic buffer containing 10% glycerol for 3 h (Miller et al., 1986).

Determination of Fractions of PDE and G-Protein Associated with Membrane Vesicles. Aliquots of the reassociated 0 PO_4/Rho and high PO_4/Rho preparations were centrifuged in a Beckman airfuge (at 100000*g*) for 15 min in order to separate the membrane-associated and soluble PDE and G-protein populations. Supernatants were subjected to SDS–PAGE and the Coomassie Blue staining intensities of the PDE and G-protein bands compared as described by Miller et al. (1986).

Assay of PDE Activity. All manipulations of rhodopsin-containing membranes were performed in complete darkness. Light-stimulated PDE activity was assayed by continuous pH monitoring according to the method of Liebman and Evanczuk (1982) as previously described (Miller & Dratz, 1984; Aton & Litman, 1984; Miller et al., 1986). A one to one relationship between H^+ produced and cGMP hydrolyzed is assumed. Rates are corrected for dark activity and expressed as moles of cGMP hydrolyzed per second per mole of rhodopsin.

Results

Rhodopsin phosphorylation in response to large fractional bleaches produces a mixture of species containing different levels of incorporated phosphate. Chromatofocusing allows the separation of these various phosphorylated species on the basis of differences in their isoelectric points. The assignment of the number of PO_4/Rho was made on the basis of the results of Aton et al. (1984). In Figure 1, lanes 1–4, equal amounts (15 μg) of rhodopsin were loaded into each lane. The isolated species that were reconstituted into PC vesicles were unphosphorylated, regenerated rhodopsin (0 PO_4/Rho; Figure 1, lane 1), rhodopsin containing two phosphates (2 PO_4/Rho; Figure 1, lane 2), and highly phosphorylated rhodopsin (high PO_4/Rho; Figure 1, lanes 3 and 4) that consisted of pooled material from chromatofocusing peaks representing ≥4 PO_4/Rho. Native rhodopsin focuses at pH 6.0, the 2 PO_4/Rho species focus as a doublet near pH 5.4, and the high PO_4/Rho species focus as a series of bands below pH 5.1. No detectable 0 PO_4/Rho is present in the isolated phosphorylated species (Figure 1, lanes 2–4). The shadowing that is visible in the high p*I* range of lane 4 does not result from contamination by unphosphorylated rhodopsin and low PO_4/Rho species. The low PO_4/Rho species resolve faster than high PO_4/Rho species; therefore, we would expect that any contaminating 0 or 2 PO_4/Rho species present in lane 4 to be visible as distinct bands and not as a shaded region. Isoelectric focusing gels were also performed following vesicle reconstitution to verify that no phosphates were lost during the reconstitution process. In all samples, the isoelectric focusing pattern was identical with that of the starting material, demonstrating no change in the state of rhodopsin phosphorylation occurs during reconstitution. A representative hypotonic protein extract is shown in Figure 1, lane 5.

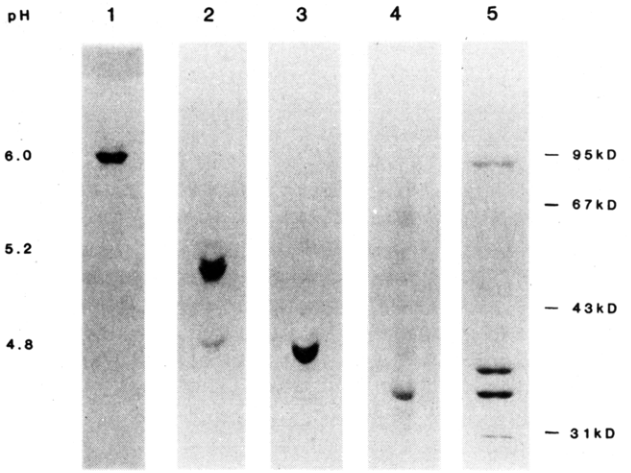

FIGURE 1: Lanes 1–4: isoelectric focusing profiles of chromatofocusing column fractions. Lane 1 contains the 0 PO_4/Rho species (p*I* = 6.0), lane 2 contains predominantly 2 PO_4/Rho species (p*I* = 5.1), lane 3 contains 4 PO_4/Rho species (p*I* = 4.8), and lane 4 contains ≥4 PO_4/Rho species (p*I* < 4.8). Each lane was loaded equally to contain 15 μg of rhodopsin. Lane 5: the hypotonic protein extract subjected to SDS–PAGE as described under Materials and Methods.

The reconstituted Rho–PC vesicles had an average final phospholipid/protein ratio of 120/1 and were shown by negative stain electron microscopy to contain predominately unilamellar vesicles of approximately 500–800-Å diameter.

The PDE activity elicited by bleaching from 0.01% to 10% of the rhodopsin present in 0 PO_4/Rho and high PO_4/Rho reconstituted vesicles is shown in Figure 2. Maximal PDE velocities ranged from 1 to 4.8 mol of cGMP hydrolyzed s^{-1} (mol of rhodopsin)$^{-1}$. Rhodopsin bleaches of 0.05% and 0.2% were required to produce half the maximum velocity of cGMP hydrolysis in the 0 PO_4/Rho and high PO_4/Rho reconstituted samples, respectively. The log–linear portion of the stimulus–response curve for the high PO_4/Rho reconstituted samples was shifted approximately 0.8 log unit toward lowered sensitivity relative to the 0 PO_4/Rho reconstituted samples. When 2 PO_4/Rho reconstituted samples were assayed, the log–linear portion of the stimulus–response curve was shifted approximately 0.4 log unit toward reduced sensitivity.

The differences in PDE activation between the 0 PO_4/Rho and high PO_4/Rho reassociations could potentially arise from a redistribution of PDE and/or G-protein into soluble forms due to the large negative charge of the high PO_4/Rho species. To test this, we sedimented 0 PO_4/Rho and high PO_4/Rho reassociated vesicles and subjected the supernatants to SDS–PAGE. Comparison of the Coomassie Blue staining intensities of the PDE and G-protein bands between the 0 PO_4/Rho and high PO_4/Rho supernatants indicated that no significant

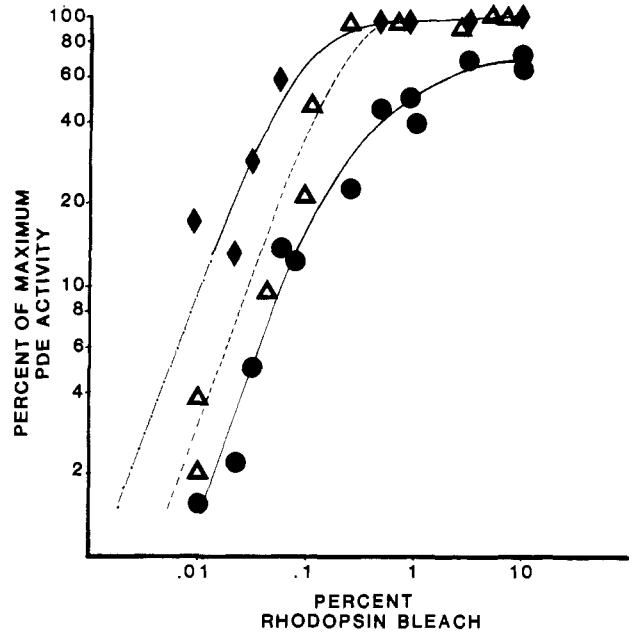

FIGURE 2: PDE stimulus–response curves for 0 PO_4/Rho (♦), 2 PO_4/Rho (△), and ≥4 PO_4/Rho (●) vesicles. Rhodopsin-containing vesicles and protein extracts were reassociated, and the light-activated PDE activity was measured as described under Materials and Methods. Data represent three separate vesicle reconstitution experiments. The V_{max} of the PDE in these experiments ranged from 1 to 4.8 mol of H^+ (mol of rhodopsin)$^{-1}$ s^{-1}. The reaction mixture initially contained 1 mM cGMP, 50 μM GTP, and 5 μM rhodopsin at pH 8.0 and 30 °C.

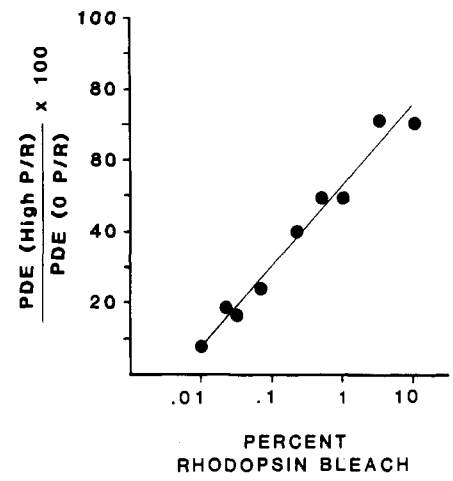

FIGURE 3: Inhibition of PDE activity in the high PO_4/Rho vesicles relative to 0 PO_4/Rho vesicles as a function of fractional rhodopsin bleach. The PDE activities of the high PO_4 curve were divided by the corresponding PDE activity of the 0 PO_4/Rho curve of Figure 2. These results are expressed as a percentage of the 0 PO_4/Rho PDE activity.

differences between the membrane-associated and soluble populations existed. Approximately 70–80% of the PDE and G-protein sediment with the membrane vesicles (data not shown).

The 0 PO_4/Rho reconstituted samples produced higher PDE velocities than high PO_4/Rho reconstituted samples at all bleaching levels tested. The relative PDE activities of the 0 PO_4/Rho and high PO_4/Rho reconstituted samples changed as a function of the fractional rhodopsin bleach, as shown in Figure 3. The difference in activity is greatest at the lowest bleaching level but becomes less pronounced as the amount of Rho* is increased with increasing flash intensities. Following a bleach of 0.01%, for example, the high PO_4/Rho reconstituted sample produces only 10% of the PDE activity observed in the 0 PO_4/Rho reconstituted sample. The percent of PDE activity in the high PO_4/Rho recombinant relative to the 0 PO_4/Rho reconstituted sample is increased to 71% following a 10% rhodopsin bleach.

Because the most pronounced inhibition of PDE activation occurred following low fractional rhodopsin bleaches, it was necessary to confirm that the perturbations on PDE activation result from rhodopsin phosphorylation and not from contributions by contaminating 48K protein. Several lines of evidence suggest that contamination by 48K protein did not significantly contribute to our observations. First, the time course of Rho* inactivation by slow thermal processes and by the rapid, 48K protein-dependent quenching is significantly different. The thermal decay of the photoactivated forms of 0 PO_4/Rho and of high PO_4/Rho described above is slow, requiring over 10 min for the inactivation of either Rho* ($\tau_{off}[1/e] \cong 400$ s, where $\tau_{off}[1/e]$ is the time required for the activity to fall to $[1/e]V_{max}$). When purified 48K is added to aliquots of the same reaction mixture (with molar ratios of 48K/Rho* as low as 2/1), the inactivation of the photoactivated form high PO_4/Rho is greatly accelerated ($\tau_{off}[1/e] \cong 60$ s; unpublished observation). Second, SDS–PAGE indicates that very little 48K protein is present in the protein extracts used in these studies. Finally, a quantitative estimate of 48K protein in the protein extracts was obtained by radiolabeling the protein extract and protein extracts "spiked" with incrementally increasing amounts of purified 48K protein with ^{125}I. On the basis of these determinations, we estimate that a maximum of one 48K protein molecule was present per 10^5 G-protein molecules and that the lowest rhodopsin bleaches produced at least a 50-fold excess of Rho* over 48K protein.

Discussion

The current investigation was undertaken in order to determine the effect of rhodopsin phosphorylation, independent of any contribution of the 48K protein, on the activation of the ROS PDE. This was accomplished by reconstituting column-purified species of phosphorylated rhodopsin into PC vesicles and reassociating this preparation with an extract of extrinsic ROS proteins, essentially free of 48K protein. This approach offered clear advantages over previously reported studies. First, by beginning with purified phosphorylated rhodopsin, we could determine the effect of rhodopsin phosphorylation over a wide stimulus range, where, in a system containing both phosphorylated and unphosphorylated rhodopsin, limiting amounts of bleached phosphorylated rhodopsin species would be overwhelmed by large pools of unphosphorylated Rho* (Miller & Dratz, 1984). Second, we were able to examine the ability of specific phosphorylated rhodopsin species to activate the ROS PDE.

The amount of PDE activation that was produced by the reconstituted rhodopsin species varied as a function of both PO_4/Rho ratio and the fraction of rhodopsin bleached. At low fractional rhodopsin bleaches and high PO_4/Rho, the PDE activity was only 10–20% of that observed for a 0 PO_4/Rho control reassociated with the same protein extract. Progressively higher fractional rhodopsin bleaches yielded up to 70% of the activity of the controls (see Figure 3). When the PDE assay data were combined into stimulus–response curves, the log–linear portions of the curves were shifted toward reduced sensitivity by about 1 log unit as the PO_4/Rho ratio was increased from 0 to ≥4. Thus, our results demonstrate that phosphorylated Rho* is capable of supporting PDE activation and that there is a graded reduction of light-stimulated PDE activity with increasing rhodopsin phosphorylation.

In a recent report, Wilden et al. (1986), using phosphory-

lated, regenerated disk membranes, also observed a reduction in PDE activation in the absence of 48K protein. However, the inhibition of PDE activation in our high PO_4/Rho recombinant vesicles is significantly more pronounced. These differences may reflect an additional contribution by small amounts of unphosphorylated and low PO_4/Rho rhodopsin species to PDE activation in the phosphorylated, regenerated disk membranes. The single reconstitution experiment reported by Wilden et al. (1986), using a pool of phosphorylated rhodopsin species, supports our observation that phosphorylated rhodopsin can stimulate PDE activation. Although these authors did not present stimulus–response curves or perform experiments to determine the effect of variable levels of rhodopsin phosphorylation on PDE activation, a comparison at a single bleach level of 0.8% (where we observed a less pronounced effect of rhodopsin phosphorylation) can be made. The level of PDE activity in our reconstituted system showed about a 30% greater inhibition than was observed by Wilden et al. (1986).

The log unit shift in sensitivity, shown in Figure 2, is comparable to that reported when PDE assays were performed on near intact ROS (Liebman & Pugh, 1982) or on disk membranes containing unphosphorylated rhodopsin reassociated with pooled isotonic and hypotonic supernatants (Miller & Dratz, 1984), assayed in the presence of ATP. In our studies 2 PO_4/Rho samples produced PDE activities that were intermediate between 0 PO_4/Rho and high PO_4/Rho samples at low fractional rhodopsin bleaches, but were identical with the 0 PO_4/Rho sample at high fractional rhodopsin bleaches. A qualitatively similar trend was observed by Miller and Dratz (1984) when the extent of rhodopsin phosphorylation was limited by proteolytically removing phosphorylation sites from rhodopsin in disk membranes prior to reassociation with combined isotonic and hypotonic protein extracts. In addition, earlier experiments from this laboratory had demonstrated up to a 50% reduction in maximal PDE activity with increasing average level of phosphorylation in disks (Aton & Litman, 1984), in agreement with the trends observed here. However, these latter experiments (Miller & Dratz, 1984; Aton & Litman, 1984) did not distinguish between contributions by rhodopsin phosphorylation and 48K protein (which is absent in the current experiments) to the ATP-dependent reduction in PDE activation. The observation that phosphorylated rhodopsin supports PDE activation is consistent with Kuhn's proposition that the 48K protein is necessary for *complete* suppression of PDE activation (Kuhn et al., 1984; Wilden et al., 1986). However, further experiments using reconstituted phosphorylated samples and 48K protein will be required to characterize the additional inhibitory role of the 48K protein.

Given the current understanding of the PDE activation, one would expect rhodopsin phosphorylation to disturb some aspect of the interaction between Rho* and G-protein. Perturbation of G-protein activation might occur during Rho*–G-protein binding, the GTP/GDP exchange reaction, or the release of the activated G-protein. Recent sedimentation studies by Kuhn and co-workers (Kuhn et al., 1984) indicate that disk membranes containing high average PO_4/Rho ratios (ca. 5.2–7 PO_4/Rho) are still capable of binding the G-protein, suggesting that rhodopsin phosphorylation does not inhibit the binding of G-protein to phosphorylated Rho*. This study did not, however, provide a quantitative measure of G-protein binding; thus a change in binding affinity sufficient to affect PDE activation cannot be ruled out. Our preliminary evidence indicates that the G-protein-dependent enhancement of metarhodopsin II formation (Emeis & Hoffman, 1981) is reduced by as much as 80% in high PO_4/Rho vesicles relative to 0 PO_4/Rho vesicles (unpublished observation), suggesting that the interaction of highly phosphorylated rhodopsin with G-protein is weakened relative to unphosphorylated rhodopsin. The apparent reduction of G-protein binding to phosphorylated Rho* is consistent with the results presented in Figure 3 that suggest the reduced affinity of G-protein for phosphorylated Rho* can be overcome by the increased potential for G-protein–Rho* interactions generated by large fractional rhodopsin bleaches (i.e., the formation of G-protein–Rho* complexes is favored by increasing the Rho* concentration through mass action).

In summary, our preparation, consisting of purified phosphorylated rhodopsin species, provide a means to evaluate the effect of rhodopsin phosphorylation on light-stimulated PDE activity. In these experiments, we observe a graded inhibition of PDE activation with increasing PO_4/Rho ratio. Furthermore, rhodopsin phosphorylation alone can inhibit at least 80–90% of the light-initiated PDE activation and hence represents a major component of the ATP-dependent shutoff observed in kinase-containing preparations.

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Complete Primary Structure of Prostatropin, a Prostate Epithelial Cell Growth Factor†

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ABSTRACT: Bovine brain prostatropin is a potent and essential mitogen for prostate epithelial cell growth. The major form of prostatropin contains 154 amino acid residues in a single amino terminally blocked chain corresponding to a molecular weight of 17 400. The amino acid sequence of the 150 carboxy-terminal residues of prostatropin was derived by Edman degradation of overlapping peptides primarily generated by cleavage at lysyl and glutamyl residues. Analysis of the amino-terminal tetradecapeptide by fast atom bombardment mass spectrometry identified the blocking group as an acetyl moiety, and tandem mass spectrometry provided the sequence of the first 12 residues. Prostatropin residues 15–154 contain the sequence of bovine brain polypeptides recently described as acidic fibroblast growth factor and class I heparin-binding growth factor. The sequence of the first 25 residues of prostatropin is acetyl-Ala-(Gly, Glu)-Glu-Thr-Thr-Thr-Phe-Thr-Ala-Leu-Thr-Glu-Lys-Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys-Lys-Pro. Reduced and carboxymethylated prostatropin exhibits mitogenic activity, suggesting that disulfide bonds among cysteine residues 30, 61, and 97 are not functionally essential. These results demonstrate by rigorous structural analysis that the brain-derived polypeptide previously described only as a mesenchymal and neuroectodermal cell mitogen is also an epithelial cell growth factor that may be involved in support of prostate hyperplasia and adenocarcinoma.

The androgen-independent proliferation of isolated epithelial cells from androgen-responsive rat prostate tumors and androgen-dependent normal prostate of rat and human requires polypeptides (prostatropins) that are concentrated in neural tissue[1] (McKeehan et al., 1984; Chaproniere & McKeehan, 1986). Two molecular forms of prostatropins were recently purified to homogeneity from bovine brain by ammonium sulfate fractionation, heparin–agarose chromatography, and reverse-phase high-performance liquid chromatography (RP-HPLC)[2] (Crabb et al., 1986). One form had a molecular weight of about 16 000 and an unblocked amino terminus, and the other form had a molecular weight of about 18 000 and a blocked amino terminus. The two forms were distributed among five chromatographic peaks and collectively consisted of about 70% blocked molecular weight 18 000 forms and about 30% unblocked molecular weight 16 000 forms. Preliminary characterization suggested that the smaller form was derived from the larger form, perhaps through proteolytic processing. Both molecular species contained regions of sequence identical with neural tissue derived, heparin-binding growth factors that have been isolated on the basis of mitogenic activity for fibroblasts and endothelial cells. Here we report the complete primary structure of the amino terminally acetylated, predominant form of bovine brain prostatropin and

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[1] W. L. McKeehan, S. Adams, and D. Fast, unpublished results.
[2] Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; ECGF, endothelial cell growth factor; EDTA, ethylenediaminetetraacetic acid; FAB-MS, fast atom bombardment mass spectrometry; Gdn-HCl, guanidine hydrochloride; HBGF, heparin-binding growth factor; PEC, (pyridylethyl)cysteine; RP-HPLC, reverse-phase high-performance liquid chromatography; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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