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Effect of Phosphorylation on Receptor Conformation: The Metarhodopsin I ↔ Metarhodopsin II Equilibrium in Multiply Phosphorylated Rhodopsin†

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ABSTRACT: The superfamily of membrane-bound receptors, which function in signal transduction by activating a guanine nucleotide binding protein or G-protein in response to agonist binding, shares a number of structural and mechanistic properties. Among these similarities is downregulation of functional activity via receptor phosphorylation. In this study, the effects of intermediate levels of phosphorylation (≥4 added phosphates per receptor molecule) on receptor conformational equilibria are examined by comparing the photochemical properties of phosphorylated and unphosphorylated rhodopsins which were incorporated separately into 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine vesicles. Postflash spectra reflecting the contributions of metarhodopsins I, II, and III (meta I, meta II, and meta III) were obtained from these samples. Deconvolution of appropriate difference spectra allowed a determination of the concentration of the photointermediates of interest. Meta II is the form of photolyzed rhodopsin which binds and activates the visual G-protein (G_t); thus, its relative abundance at equilibrium and temporal stability are important parameters in determining the efficiency of visual signal transduction. The effects of pH and temperature on the meta I ↔ meta II equilibrium constant (K_{eq}) and the rate of decay of meta II to meta III were examined for the reconstituted phosphorylated and unphosphorylated rhodopsin samples. K_{eq} was essentially unaffected by phosphorylation when measured at pH 7.0 and 8.0 and 20 and 37 °C. The decay time (lifetime) of meta II → meta III had a value of approximately 4.7 min in both phosphorylated and unphosphorylated samples. These results demonstrate that phosphorylation does not affect either the kinetics of the depletion of meta II or the extent of formation of meta II. Therefore, it would appear that receptor phosphorylation affects downregulation of signal transduction activity at the level of the G-protein–receptor interaction and not by alteration of intrinsic receptor conformational properties involving the activated, functional form of the receptor.

The visual pigment rhodopsin is a member of the superfamily of membrane-bound receptors, which share a highly conserved structural motif, consisting of seven transmembrane helices joined by hydrophilic loops, and participate in signal transduction by binding a G-protein[1] and catalyzing the exchange of bound GDP for GTP. Signaling desensitization, or downregulation, via receptor phosphorylation has been reported for rhodopsin (Liebman & Pugh, 1980; Aton & Litman, 1984; Miller et al., 1986), the β_2-adrenergic receptor (Strasser et al., 1986; Benovic et al., 1986), the M2 muscarinic acetylcholine receptor (Kwatra & Hosey, 1986; Kwatra et al., 1987, 1989), the chemotactic cAMP receptor of the slime mold *Dictyostelium discoideum* (Klein et al., 1985), and the α-mating factor receptor of the yeast *Saccharomyces cerevisiae* (Reneke et al., 1988). Three members of the family of kinases specific for G-protein-coupled receptors have been identified and studied: rhodopsin kinase (Wilden & Kuhn, 1982), β-adrenergic receptor kinase (Benovic et al., 1989), and

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[1] Abbreviations: G-protein, guanine nucleotide binding protein; PDE, phosphodiesterase; ROS, retinal rod outer segment; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PIPES, piperazine-*N*,*N*′-bis(ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; DTPA, diethylenetriaminepentaacetic acid; meta I, metarhodopsin I; meta II, metarhodopsin II; meta III, metarhodopsin III; G_t, G-protein of the visual system transducin; K_{eq}, equilibrium constant for meta I ↔ meta II; rho*, catalytically active form of photolyzed rhodopsin, spectroscopically identifiable as meta II.

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β-adrenergic receptor kinase 2 (Benovic et al., 1991). These findings suggest that downregulation of receptor function via phosphorylation of the agonist-stimulated receptor by a receptor-specific kinase may be a general mechanistic motif in G-protein-mediated signal transduction pathways.

Rhodopsin is unique among G-protein-activating receptors in that one can monitor the conformational changes associated with its activation spectroscopically, due to the presence of the retinal moiety. Thus, rhodopsin makes an ideal model for studying the effect of phosphorylation on the ability of a receptor to adopt the required G-protein-activating conformation. Although rhodopsin differs from other members of this family in that its ligand is covalently bound, a close similarity in terms of receptor conformational changes upon ligand binding may be drawn. In the dark, the 11-cis form of the retinal chromophore acts as an antagonist. Absorption of a photon converts 11-*cis*-retinal to the all-trans form, which functions as an agonist. Following photoisomerization, the "agonist-bound" receptor undergoes a series of conformational changes to form rho*, the catalytically active form of rhodopsin, which binds and activates G_t. The absorption spectrum of rhodopsin is strongly influenced by the interaction of retinal with the amino acid side chains in the retinal binding pocket (Honig & Ebrey, 1982). Isomerization of the retinal chromophore and subsequent changes in protein conformation produce distinctive shifts in the retinal absorption spectrum. A large body of work has established that rho* is synonymous with meta II, the most blue-shifted metarhodopsin photointermediate (Bennett et al., 1982; Emeis et al., 1982; Kibelbek et al., 1991). Meta II (λ_{max} = 380 nm) is formed within about 1 ms of photoexcitation and exists in conformational equilibrium with a catalytically inactive photointermediate, meta I (λ_{max} = 478 nm). Thus, chromophore photoisomerization in the visual transduction pathway leads to the formation of the rhodopsin conformation necessary for G-protein binding; this is functionally equivalent to the receptor conformational change resulting from a diffusible ligand binding and activating a receptor. The spectral changes of rhodopsin and its photointermediates make it possible to monitor closely the antagonist-bound (unbleached rhodopsin), agonist-bound yet catalytically inactive (photointermediates other than meta II with an *all-trans*-retinal chromophore), and catalytically active agonist-bound (rho*/meta II) forms of the receptor.

In the visual signal transduction system many details of agonist-stimulated phosphorylation of rhodopsin have been elucidated. Phosphorylation is thought to be the key regulatory step in the rapid inactivation of rho* (Liebman & Pugh, 1980). This model is supported by the observations that light-stimulated PDE activity decreases with increasing rhodopsin phosphorylation (Miller et al., 1986), and light-stimulated PDE activity is rapidly quenched when ATP and rhodopsin kinase are present (Liebman & Pugh, 1979, 1980), but not in the presence of ATP alone (Sitaramayya & Liebman, 1983). Rhodopsin kinase sequentially adds up to 9 phosphate groups to the cytoplasmic surface of light-activated rhodopsin (Wilden & Kuhn, 1982; Aton et al., 1984; McDowell et al., 1985). Proteolysis studies, which removed portions of the C-terminal tail of rhodopsin, have established that phosphorylation of this region of rhodopsin is critical in desensitization (Miller & Dratz, 1984). Receptor inactivation is also aided by the binding of arrestin to phosphorylated rhodopsin (Kuhn et al., 1982; Wilden et al., 1986; Bennett & Sitaramayya, 1988; Schleicher & Hofmann, 1989).

One possible mechanism for the inactivation of visual signal transduction by rhodopsin phosphorylation is that the addition of phosphate groups alters the stability of meta II relative to catalytically inactive, agonist-bound conformations, resulting in a reduction in the steady-state concentration of meta II. Meta III (λ_{max} = 465 nm) is the intermediate which follows meta II in the photointermediate cascade of bleached rhodopsin and, like meta I, is catalytically inactive. A reduction in the stability of meta II relative to meta III would cause meta II to decay to meta III more rapidly. On the other hand, a reduction in meta II stability relative to meta I would shift the meta I ↔ meta II equilibrium toward meta I. Either of these modifications would have the effect of lowering the concentration of the activated receptor, meta II/rho*, thereby decreasing the level of light-stimulated G_t activation and resultant PDE activity. In these experiments, we have examined the effect of phosphorylation on the conformational stability of meta II by comparing the photoresponses of phosphorylated and unphosphorylated rhodopsin samples reconstituted separately into phospholipid vesicles. The phosphorylated sample was a pool of rhodopsins which contained ≥4 phosphate groups per rhodopsin, with an average phosphorylation level of 5–6. The photochemical characteristics of the two samples were compared in terms of the K_{eq} for the meta I ↔ meta II equilibrium and decay time (lifetime) for the loss of meta II as it converts to meta III.

MATERIALS AND METHODS

Preparation, Characterization, and Purification of Phosphorylated Rhodopsin. Rhodopsin in ROS suspensions, prepared from frozen bovine retinas, was phosphorylated, purified, characterized in terms of phosphorylation state, and incorporated into pure phospholipid vesicles. Briefly, a homogenized ROS suspension was bleached with white light in the presence of added ATP to yield a mixture of phosphorylation states including 0 phosphates per rhodopsin. This sample was regenerated with an excess of 11-*cis*-retinal, then solubilized in ocytl β-D-glucoside, and finally purified on a concanavalin A–Sepharose column. The fractions containing purified rhodopsin were pooled, concentrated, and applied to a chromatofocusing column to separate differentially phosphorylated species of rhodopsin (Aton et al., 1984). Ampholytes from the chromatofocusing elution buffer were removed from each pool of phosphorylated species via an additional concanavalin A–Sepharose column. The resulting samples were characterized in terms of purity with isoelectric focusing gels following the procedure of Aton and Litman (1984). Column pools containing phosphorylated species of ≥4 phosphates per rhodopsin were combined to yield the phosphorylated rhodopsin sample, and both phosphorylated rhodopsin and unphosphorylated rhodopsin were then reconstituted into large unilamellar POPC vesicles following the dilution reconstitution procedure described by Jackson and Litman (1985).

Spectrophotometric Measurements. All spectrophotometric measurements were carried out with a Hewlett-Packard 8452A diode array UV/vis spectrophotometer. Values of the meta I ↔ meta II K_{eq} were calculated using deconvolved difference absorption spectra of meta I–meta II equilibrium mixtures following the detailed procedures of Straume et al. (1990). Vesicle samples were suspended in buffered solutions which included 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT, and 50 μM DTPA. Samples adjusted to pH 7.0 included 10 mM PIPES, and samples adjusted to pH 8.0 included 10 mM Tris. Spectra were recorded of (1) the initial sample, (2) the sample immediately following ca. 25% bleach by a green-filtered flash (500 ± 20 nm band-pass filter) from

Table I: Phosphorylated Rhodopsin: Final Distribution of Phosphorylated Species Incorporated into POPC Vesicles

no. of phosphates	4	5	6	7	8	9
%	26	41	21	0	7	5

a camera strobe, (3) the sample following addition of 2 M hydroxylamine to a final concentration of 30 mM (to convert all bleached chromophores to retinal oxime), and (4) the sample after complete bleaching. All spectra were acquired in 0.2 s, which typically bleached less than 0.3% of the sample. Difference spectra of meta I ↔ meta II equilibrium mixtures were obtained by subtracting curve 1 from curve 2 and adding to the resulting difference spectrum the spectrum of the bleached rhodopsin. K_{eq} was calculated using molar extinction coefficients of 38 000 M^{-1} cm^{-1} for meta II and 44 000 M^{-1} cm^{-1} for meta I (Applebury, 1984).

The decay time of meta II to meta III was determined according to the detailed procedures outlined in Kibelbek et al. (1991). A series of 15–20 scans were collected at 45-s to 5-min intervals beginning ~5 s after the bleaching flash. Only the first (~5-s postflash) and last (>30-min postflash) postflash corrected difference spectra were explicitly fit with the sum of two symmetric quasi-Gaussian band shapes. The first postflash spectrum was modeled as an equilibrium mixture of meta I and meta II, and the last postflash spectrum was modeled as a mixture of *N*-retinylidene oxime (NRO) and meta III, which are the decay products of the meta I–meta II equilibrium at 20 °C, pH 8.0 (Blazynsky & Ostroy, 1981, 1984). All corrected difference spectra from intermediate times were fit with linear combinations of the derived meta I–meta II and meta III–NRO absorbance profiles. The resulting peak heights for meta II and meta III were used to determine the rate of decay of meta II and the rate of formation of meta III, respectively. Decay of meta II and the rise of meta III were both modeled as single-exponential processes.

RESULTS

Previous studies have demonstrated that rhodopsin with ≥4 added phosphates has an enhanced inhibitory effect on light-stimulated PDE activity relative to rhodopsin with only 2 added phosphates (Miller et al., 1986). Thus, in the present investigation, we have focused on the photochemical properties of a rhodopsin pool having ≥4 added phosphates. The light-induced phosphorylation of rhodopsin in vitro produces a distribution of phosphorylated species, which can be shifted by altering the concentration of ATP and/or duration of exposure to light. Each added phosphate further decreases the isoelectric point of rhodopsin; this characteristic allows variably phosphorylated rhodopsins to be separated by chromatofocusing. The number of added phosphates per rhodopsin was determined from the resulting chromatogram on the basis of the results of Aton et al. (1984). Column fractions containing ≥4 added phosphates were pooled and reconstituted. An example of the resulting distribution of phosphorylated species is given in Table I, where the average number of added phosphates in this preparation was 5.6.

The possibility that phosphorylation of rhodopsin alters the equilibrium concentration of meta II was investigated spectrophotometrically at 20 and 37 °C and pH 7.0 and 8.0. Individual meta I and meta II absorbance bands were deconvolved from corrected difference spectra of their equilibrium mixture, and values of the meta I ↔ meta II equilibrium constant, K_{eq}, were calculated using their respective extinction coefficients. Examples of deconvolved spectra for both unphosphorylated and phosphorylated rhodopsin at pH 8.0 and 37 °C are shown in panels A and B of Figure 1, respectively. Band shapes and peak locations of both meta I and meta II were identical within experimental error in both phosphorylated and unphosphorylated rhodopsin at all temperatures and pH's studied. In phosphorylated rhodopsin, K_{eq} exhibited the same dependence on both pH and temperature as in unphosphorylated rhodopsin; it was reduced by increased pH and by decreased temperature, as shown in Table II. Under all pH and temperature conditions investigated, K_{eq} was approximately the same in phosphorylated and unphosphorylated rhodopsin.

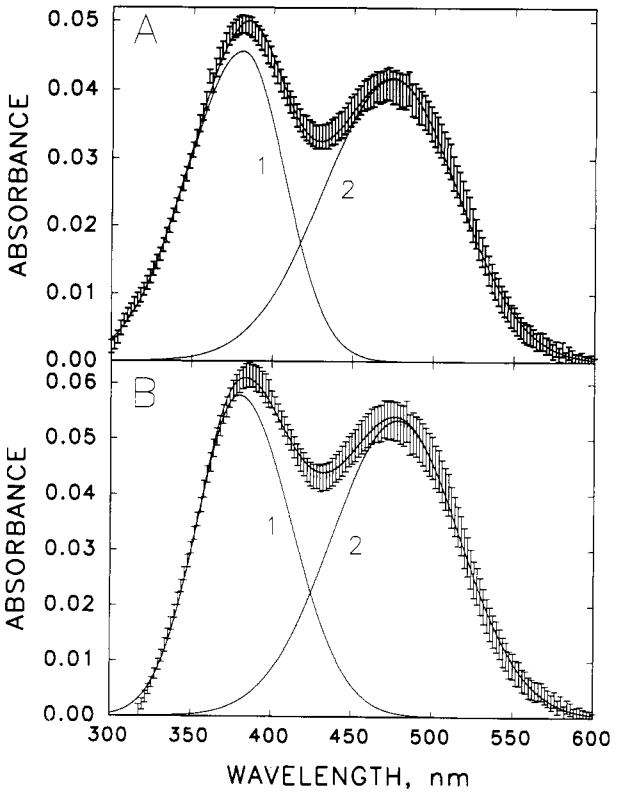

FIGURE 1: Corrected difference spectra of meta I ↔ meta II at 37 °C, pH 7.0. Spectral data are shown as points with error bars. Error bars represent the uncertainty reported by the spectrophotometer which was propagated during spectral subtraction and correction. The smooth curve through the data is the total fit, and individual meta I (1) and meta II (2) bands are also shown. Panels: (A) unphosphorylated rhodopsin (meta I λ_{max} = 474 ± 2 nm, meta II λ_{max} = 382 ± 1 nm); (B) phosphorylated rhodopsin (meta I λ_{max} = 478 ± 3 nm, meta II λ_{max} = 381 ± 1 nm).

Table II: Values of Meta I ↔ Meta II K_{eq} for Phosphorylated and Unphosphorylated Rhodopsin in POPC Vesicles[a]

conditions	unphosphorylated rhodopsin	phosphorylated rhodopsin
pH 8.0, 20 °C	0.33 ± 0.03	0.36 ■ 0.03
pH 7.0, 20 °C	0.70 ± 0.04	0.78 ± 0.05
pH 8.0, 37 °C	1.32 ± 0.07	1.25 ± 0.08
pH 7.0, 37 °C	1.65 ± 0.13	1.55 ± 0.12

[a] Uncertainties represent one standard deviation. Standard deviations of individual data sets were propagated for determination of all final values of K_{eq} and the standard deviation. Conditions: pH 8.0, 20 °C, n = 7; pH 7.0, 20 °C, n = 3; pH 8.0, 37 °C, n = 3; pH 7.0, 37 °C, n = 2.

In order to determine the effect of phosphorylation of rhodopsin on the temporal stability of meta II, the rate of transition from a predominantly meta I ↔ meta II equilibrium to a meta II ↔ meta III equilibrium was determined for phosphorylated and unphosphorylated rhodopsin at 20 °C, pH 8.0. We have previously shown that meta III is in

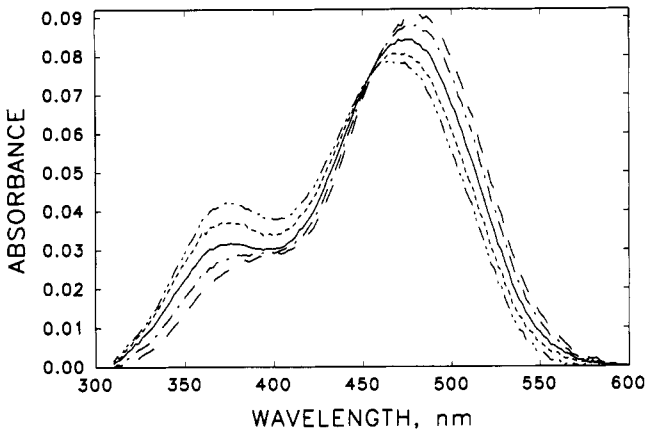

FIGURE 2: Five of the series of 20 corrected difference spectra acquired with a single phosphorylated rhodopsin sample. Postflash times were 3 s (– –), 45 s (•–•), 2.5 min (—), 7.5 min (- - -), and 17 min (•—•).

equilibrium with meta II, but the ratio of meta III to meta II is ≥20 for the equilibrium mixture at pH 8.0, 20 °C (Kibelbek et al., 1991). A series of ~20 corrected difference spectra were collected from 3 s to 40 min postflash for each sample. Five of the series of ~20 corrected difference spectra collected from a phosphorylated rhodopsin sample are shown in Figure 2. The isosbestic point resulting from the transition from one equilibrium mixture to a second equilibrium mixture is clearly evident at 452 nm in Figure 2. These corrected difference spectra were considered to be mixtures of meta I, meta II, meta III, and NRO (Blazynski & Ostroy, 1981, 1984); however, spectra of the type shown in Figure 2 were not independently deconvolved into four separate absorption bands. Rather, spectra from intermediate times were fit with linear combinations of the meta I–meta II and meta III–NRO absorption profiles derived from the 3-s and ~40-min postflash spectra, respectively. The decrease in the peak height of the derived meta II band with time was well fit with a single exponential for both phosphorylated and unphosphorylated samples, as shown in panels A and B of Figure 3, respectively. Increase in the peak height of the derived meta III band with time was also well fit with a single exponential for both samples (fits not shown). The resulting time constants for meta II decay and meta III growth for phosphorylated and unphosphorylated rhodopsin are given in Table III. Within experimental error rhodopsin phosphorylation had no effect on either decay of meta II or rise of meta III.

DISCUSSION

Phosphorylation of membrane-bound receptors has been shown to downregulate signal transduction activity in a number of G-protein-coupled pathways. Receptor phosphorylation could exert its effect either via deactivation of catalytically active receptors by altering their conformation or by interfering with binding, activation, or release of G-protein by the receptor. In this study, the former possibility has been examined by taking advantage of the fact that the catalytically active form of rhodopsin, the equivalent of an agonist-bound receptor, is directly observable spectroscopically. The sample examined in the present work represents an intermediate level of phosphorylation; ~90% of the phosphorylated sample had 4, 5, or 6 added phosphates and an average level of 5–6 phosphates per rhodopsin. We find that phosphorylation at this level had no effect on the equilibrium concentration and temporal stability of meta II, the photoactivated form of rhodopsin which participates in signal transduction. Bleached phosphorylated rhodopsin can be regenerated by the addition of

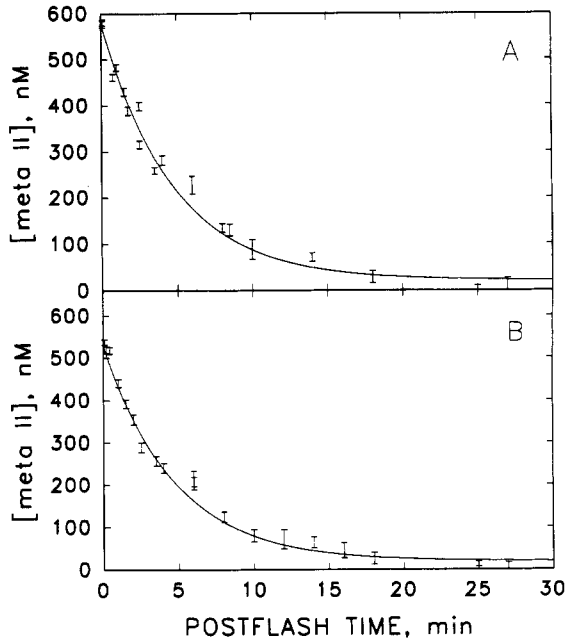

FIGURE 3: Decay of meta II in phosphorylated (A) and unphosphorylated (B) rhodopsin. Meta II concentrations were calculated from peak heights of the derived meta II absorption bands using $\epsilon_{\text{meta II}}$ = 38 000 cm^{-1} M^{-1} (Applebury, 1984), as described under Materials and Methods (Spectrophotometric Measurements).

Table III: Time Constants of Meta II Decay and Meta III Rise[a]

	τ_{decay}, meta II (min)	τ_{rise}, meta III (min)
unphosphorylated rhodopsin	4.7 ± 0.4	4.6 ● 0.3
phosphorylated rhodopsin	4.7 ± 0.6	4.5 ± 0.5

[a] Errors represent one standard deviation; n = 3 for phosphorylated and unphosphorylated rhodopsin.

11-*cis*-retinal (Aton et al., 1984). This shows that phosphorylation does not interfere with ligand binding by the unoccupied receptor, opsin. Thus, these studies demonstrate that phosphorylation does not alter the ability of rhodopsin to bind ligand and adopt the catalytically active, agonist-bound conformation. In addition, they strongly support the suggestion that rhodopsin phosphorylation downregulates visual signal transduction by disrupting some step in the activation of G_t by rhodopsin and not by affecting the concentration of the activated receptor. This conclusion is supported by preliminary results in our laboratory, which indicate that phosphorylation, at the level examined in this study, increases the dissociation constant of meta II–G_t by an order of magnitude.

The high level of structural and functional similarity within the superfamily of G-protein-activating membrane receptors suggests that the conclusions reached from our studies of the effect of phosphorylation on rhodopsin conformational equilibria may hold for the other members of this receptor superfamily, whose agonists are free ligands. Thus, an implication of the present work is that receptor phosphorylation in general does not reduce the ability of the receptor to bind agonist and adopt a catalytically active conformation once agonist is bound. We therefore suggest that the crucial determinant in terms of phosphorylation-induced downregulation of signal transduction activity is disruption of the receptor–G-protein interaction.

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