Diffusible Ligand All-trans-retinal Activates Opsin via a Palmitoylation-dependent Mechanism

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Kristina Sachs, Dieter Maretzki, Christoph K. Meyer, and Klaus Peter Hofmann‡

From the Institut für Medizinische Physik und Biophysik, Universitätsklinikum Charité, Humboldt-Universität, D-10098 Berlin, Germany

In rhodopsin's function as a photoreceptor, 11-cis-retinal is covalently bound to Lys296 via a protonated Schiff base. 11-cis/all-trans photosomerization and relaxation through intermediates lead to the metarhodopsin II photoproduct, which couples to transducin (Gt). Here we have analyzed a different signaling state that arises from noncovalent binding of all-trans-retinal (atr) to the aporeceptor opsin and enhances the very low opsin activity by several orders of magnitude. Like with metarhodopsin II, coupling of Gt to opsin-atr is sensitive to competition by synthetic peptides from the COOH termini of both Gtα and Gtγ. However, atr does not compete with 11-cis-retinal incorporation into the Lys296 binding site and formation of the light-sensitive pigment. Blue light illumination fails to photorevert opsin-atr to the ground state. Thus noncovalently bound atr has no access to the light-dependent binding site and reaction pathway. Moreover, in contrast to light-dependent signaling, removal of the palmitoyl anchors at Cys322 and Cys323 in the rhodopsin COOH terminus impairs the atr-stimulated activity. Repalmitoylation by autoacetylation with palmitoyl-coenzyme A restores most of the original activity. We hypothesize that the palmitoyl moieties are part of a second binding pocket for the chromophore, mediating hydrophobic interactions that can activate a large part of the catalytic receptor/G-protein interface.

The retinal photoreceptor rhodopsin absorbs photons via a chromophoric ligand, 11-cis-retinal, to trigger the amplifying cascade of vision. The light-sensitive ground state conformation, with a λmax = 500 nm, is stabilized by a salt bridge between the protonated Schiff base bond of the 11-cis-retinal to Lys296 and its counterion at Glu134 (1). Light-induced 11-cis/all-trans isomerization of the retinal and subsequent relaxation processes result in protonation-dependent conformations of the receptor identified as “metarhodopsin” photointermediates (for details, see Refs. 2 and 3). Metarhodopsin II (Meta II,1 λmax = 380 nm) is distinguished by its deprotonated Schiff base linkage and broken Lys296/Glu134 salt bridge. The signaling state for Gt arises from Meta II through proton uptake from solution (4), with the conserved Glu134 as the likely proton acceptor. The apparent pK for this protonation is shifted to pH values higher than the intrinsic pK of a Glu residue, reflected in pH-rate profiles for activation of both Gt (1) and rhodopsin kinase (“forced protonation” (5)). Protonated Meta II catalyzes nucleotide exchange in Gt at a high rate (for review, see Refs. 2, 6, and 7). Each catalytic coupling of Gt requires interaction via the COOH termini of both the α and γ subunits (8).

This study deals with a fundamentally different class of signaling states. They are generated without light, by mere addition of the diffusional agonist, all-trans-retinal (atr), to the empty aporeceptor, opsin. Free opsin arises from the spontaneous decay of the Meta intermediates by hydrolysis of the deprotonated Schiff base linkage between Lys296 and atr (9). It has a measurable constitutive activity in vitro, in the order of 10⁻⁶ of Meta II (10). Reversible Schiff base formation is seen with peripheral lysine groups of opsin, leading (preferentially at pH > 8) to reversible “pseudo-photoproducts” (11, 12). These products interact with arrestin and rhodopsin kinase, but an interaction with Gt is not measured. Activity toward Gt arises from noncovalent interaction of atr with the aporeceptor. This opsin-atr complex is several orders of magnitude more active than the empty apoprotein but still less active than Meta II (at neutral pH and equimolar ratio (1, 5, 12, 13).

The weaker activation capacity of the reversible complex is reflected in a lower pK for forced protonation (1, 3, 5, 7). Atr recombines with reductively permethylated (PM)-opsin, lacking free lysine side chains with the exception of the original binding site, Lys296. Atr forms an active complex with PM-opsin, although no covalent Schiff base bond is detectable by acid trapping (12). This provides evidence that the opsin-atr activity arises from noncovalent chromophore/protein interaction, rather than from a Meta II-like deprotonated Schiff base bond. Moreover, atr does not compete with 11-cis-retinal during regeneration of PM-opsin to PM-rhodopsin, suggesting that even the binding site might be different (12).

The formation of an active complex between opsin and the photolyzed chromophore, atr, may play an important role in vivo under conditions of intense illumination. After the decay of Meta II, atr bound to opsin may keep the rod cell in noisy and/or desensitized states (14).

A new property of the opsin-atr activity, described in this study, is its dependence on palmitoylation. Generally, the S-palmitoylation of heptahelical membrane receptors may provide a structural anchor for the carboxyl-terminal tail. In rhodopsin, the two palmitoyl anchors at cysteines 322 and 323 may help to stabilize the structure of the putative fourth cytoplasmic loop (15, 16). Protein S-palmitoylation of rhodopsin was found in vivo and also after autoacylation with fatty acid acylcoenzyme A in vitro (17). The fatty acid S-acylation is specific for palmitate incorporation (18). In previous functional tests, chemical depalmitoylation of membrane-bound rhodopsin in...
the dark had no effect on the light-dependent binding of G, but rather increased its GTPase activity (19), and replacement by protein engineering of the cysteines by serines did not change G activation by dodecyl maltoside- purified rhodopsin (20).

Thus rhodopsin has two faces, that of a photoreceptor and that of a chemical receptor for the diffusible agonist, atr. We will investigate some aspects of retinal/protein and protein/ protein interaction, with emphasis on the role of palmitoylation.

**EXPERIMENTAL PROCEDURES**

Materials—Atr was purchased from Sigma. 11-cis-Retinal was a generous gift from Rosalie Crouch and the National Eye Institute, USA. The concentrations of the retinoids were determined spectrophotometrically at 380 nm using the extinction coefficients for atr of 43.400 M⁻¹ cm⁻¹ and all-trans-retinal of 24.400 M⁻¹ cm⁻¹. [3H]Palmitic acid was purchased from Amersham Pharmacia Biotech. Bovine serum albumin (BSA, essentially fatty acid-free) was obtained from Sigma. Amino acid sequences for the peptides were as described in (8): G α (340–350), IKENLKDCGLF; G α (50–71)-farnesyl, EPDLVKGPEDPKFKEKLGCC-farnesyl; G α (340–350) control, IRENLKDGCFL; G γ (50–71) control.

Preparation of Rod Outer Segments (ROS) and Membranes—Bovine ROS were prepared from fresh, dark-adapted retinas according to (21). All centrifugation steps were carried out at 4 °C, 30,000 x g for 20 min. Hypotonically washed ROS membranes were prepared by removing soluble and membrane-associated proteins by repetitive washes of ROS in low ionic strength buffer, pH 7.9, containing 5 mM Pipes and 1 mM EDTA. Hypotonically washed ROS membranes were prepared by extracting ROS with the hypotonic buffer, containing 5 mM urea as described previously (23), and washed with isotonic BTP buffer (130 mM NaCl, 20 mM bis-tris-propane, pH 7.5, 1 mM MgCl₂). All membrane suspensions were stored at −80 °C in BTP buffer containing 0.3 mM sucrose. Opsin was prepared according to (24), with modifications. Aliquots of ROS or washed membranes (about 20 mg of rhodopsin) were thawed and vortexed in 100 mL of ice-cold buffer, containing 10 mM sodium phosphate buffer, pH 6.5, containing fatty acid-free BSA (2%). The suspension was frozen for 10 min on ice with orange light. Membranes were pelleted by centrifugation and resuspended in 10 mM sodium phosphate buffer, pH 6.5, containing 5 mM urea (50 mL) with a glass/as glass Potter homogenizer. After 10 min of incubation on ice, 50 mL of phosphate buffer (100 mM, pH 6.5) containing fatty acid-free BSA (2%) was added to the suspension, which was vortexed intensively before centrifugation. Retinal oxime was extracted from the membrane-bound opsin by four washes with the same buffer containing fatty acid-free BSA. Subsequently, four washes were carried out with the buffer alone to remove BSA. Finally, the opsin membranes were washed with the isotonic BTP buffer, pH 7.5, and resuspended in the same buffer containing 0.5 mM sucrose.

Depalmitoylation of Rhodopsin and Opsin—Washed ROS membranes or urea-treated ROS membranes (10 mg of rhodopsin) were incubated in the dark with diethiothreitol (20 mM) in 50 mM Tris buffer, pH 8.6, containing 3 mM EDTA and 0.3 mM sucrose, for 16 h at room temperature to reduce the palmitoyl-cysteines. The diethiothreitol-treated membranes were washed four times with isotonic BTP buffer, pH 7.5, and stored as described above. Depalmitoylated opsin was prepared from these membranes following the protocol for opsin.

To estimate the extent of deacylation, free SH groups were determined according to (25). After incubation of about 1 µM rhodopsin or opsin with 10 µM 4,4′-dithiobis-pyridine (3, 20 °C), the absorption change at 324 nm was used to measure the released 4-thiopyridine (ε = 21,000 cm⁻¹ M⁻¹). Depalmitoylation was calculated from the differences of the reactive SH groups modified/mol of deacylated rhodopsin/opsin compared with those of the untreated controls in the dark. Preparations with the stoichiometric ratio of about two reactive SH groups/palmitoyl- and four depalmitoylated rhodopsin or opsin were used for these studies.

**RESULTS**

**Peptides Interfere with G Activation by the Opsin-Atr Complex**—As we reported recently, COOH-terminal peptides from both the G(Aγ) subunit, G(Aγ) (50–71)-farnesyl, and the G(α) subunit, G(α) (340–350), compete with G(α) holoprotein for interaction with light-activated Meta II (8). The fluorescence spectroscopy results in Fig. 1A show basically the same competition for opsin-atr, however, with higher efficiency. Even the native peptide G(α) (340–350), for which an effect was hardly detectable with Meta II, shows clear inhibition. Both G(α) (340–350) high affinity analog (Fig. 1A, inset) and G(γ) (50–71)-farnesyl are more potent than with Meta II by a factor of 10–15 (8). A quantitative comparison of peptide competition data between Meta II and opsin-atr is difficult because the G activation rate in the fluorescence assay is saturated when the content of active rhodopsin in the disc membrane exceeds 20% of the total (12, 30) (compare Fig. 4, discussed below). What we can state, however, is that the G(α) and G(γ) COOH-terminal sequences are involved in Meta II and in opsin/atr interactions. As in the case of Meta II, control peptides without farnesyl do not inhibit G activation (Fig. 1B, inset).

**Atr Does Not Compete with 11-cis-Retinal for Rhodopsin Reconstitution**—We have reported previously that atr, when it functions with reductively permethylated opsin preparations (12), does not compete with 11-cis-retinal for binding to the active site Lys236. We repeated this experiment with native, unmodified opsin under conditions of substoichiometric 11-cis-retinal (molar ratio 11-cis-retinal/opsin = 1:2). As Fig. 2 shows, atr even accelerates the uptake of 11-cis-retinal. The positive control is given by β-ionone, which inhibits opsin regeneration, as expected. The data show that the 11-cis-retinal binding site Lys236 is not disturbed by atr in the mode in which it activates the receptor, suggesting that atr does not occupy the active site.
Photoregeneration to Rhodopsin Does Not Occur from the Opsin-atr Complex—

Photoregeneration studies of the opsin-atr complex were performed to test the formation of a functional covalent Schiff base with Lys296 when opsin is incubated with atr. In Meta II, where the Schiff base is deprotonated, a flash of blue light reisomerizes the retinal back to a cis conformation. This forces reprotonation of the Schiff base and drives rhodopsin back to the ground state. Reprotonation of the Schiff base can be monitored by changes in the absorption of light at 543 nm (31). In the experiments shown in Fig. 3, the control with rhodopsin was carried out with an illuminated preparation that was regenerated from opsin membranes and 11-cis-retinal. It demonstrates the behavior known from purified rhodopsin and hypotonically washed membranes (data not shown).

The opsin-atr samples do not produce any measurable reprotonation signal, implying that the photoreversion pathway from Meta II to rhodopsin is not accessible for atr in the complex with opsin.

Effect of Depalmitoylation on the Activity of the Opsin-atr Complex—
The data in Fig. 4 confirm previous analyses that receptor palmitoylation has only a minor influence on light-dependent Gt activation (19, 20). As shown in the inset, this is also true for conditions of limiting 11-cis-retinal. This indicates that the lack of an effect of depalmitoylation is not caused by a saturation of the activation rate by the densely packed rhodopsin in disc membranes. In contrast to Meta II, the opsin-atr activity exhibits a strong dependence on the presence of receptor palmitoylation. When the palmitoyl modifications on cysteines 322 and 323 are removed by cleavage of the thioester...
Opsin Activation by All-trans-retinal

In its well studied function as a photoreceptor, rhodopsin absorbs photons via the chromophoric ligand, 11-cis-retinal. Photoactivation may be understood as the light-induced replacement, through photochemical isomerization, of the bound inverse agonist 11-cis-retinal by the agonist atr. In contrast, the exogenous addition of atr to the aporeceptor leads to a noncovalent complex that is less active than Meta II but several orders of magnitude more active than the empty opsin. In this study we have explored some properties of this activity.

The Opsin-Atr Complex Is a Form of the Receptor with an Intrinsically Low Activity—A prerequisite for the atr-induced activity is the binding of the ligand to a site from which the signal is transmitted to the interaction domain with the G protein. The most obvious explanation would be that the exogenously added chromophore binds reversibly to the original Lys296 binding site. Such a mechanism has indeed been found in constitutively active mutants (32, 33). In all mutants studied, the data were consistent with a two-state model (34), which predicts that increased constitutive activity results in increased atr-induced activity (35).

The data presented in this work reveal that reversible binding to the original site does not occur in wild type opsin to any measurable degree. Although a substantial activity of opsin-atr toward Gt is measured, it could still result from an equilibrium in constitutively active mutants (32, 33). In all mutants studied, there was a small amount of a Meta II-like species would be hard to determine by direct spectroscopy.

However, properties of the Gt activation argue for opsin-atr representing a separate form of the active receptor with intrinsically low activity. These include:

1. atr recombines not only with native opsin but also with reductively PM-opsin, lacking free lysine side chains with the exception of the original blocked binding site Lys296. In this situation, one does not find any measurable Schiff base formation with atr, although a substantial Gt activation (12) or phosphorylation of opsin by rhodopsin kinase (5) is measured.

2. Removal of the palmitoyl anchors from the COOH-terminal cysteines does not measurably affect the activity of Meta II but has a strong inhibitory effect on the activity of opsin-atr (Fig. 4, discussed below).

We thus conclude that the rod photoreceptor is capable of two very different types of activity, one Meta II-like, one opsin-like. Available evidence indicates that in this complex, atr is bound noncovalently (12).

Chromophore/Protein Interaction in the Opsin-Atr Complex Is Different from Meta II—The data presented have corroborated the data of Daemen (36) and our earlier finding (12) that atr, under conditions where it activates opsin, does not compete with 11-cis-retinal when the latter regenerates the aporeceptor to rhodopsin. We thus conclude that the overlap between the binding domains for the chromophore in the two modes of action is negligibly small. Moreover, atr has a supporting effect on regeneration of opsin to rhodopsin, indicating a positive allosteric effect of atr (Fig. 2). Thus, atr paradoxically favors...

Fig. 4. Activation of Gt by Meta II or opsin-atr complex: influence of receptor palmitoylation. Meta II- (panel A) or opsin-atr complex (panel B) catalyzed GTP$\gamma$S uptake by Gt, as measured by the increase in fluorescence intensity relative to the respective dark controls at increasing molar ratios of atr/opsin. The inset shows the relative rates of Gt activation by Meta II and opsin-atr complexes formed from 0.5 μM opsin and 1.0 μM atr (panel B). The inset shows the rate of Gt activation by palmitoylated (open squares) or depalmitoylated (open circles) opsin regenerates with increasing substoichiometric amounts of atr/opsin molar ratios (data are from least seven rhodopsin, opsin, or the corresponding depalmitoylated preparations, each analyzed by duplicate or triplicate fluorescence assays). In all experiments, the Gt concentration was 1 μM, and the GTP$\gamma$S concentration was 5 μM.

bond with dithiothreitol (see “Experimental Procedures”), the rate of Gt activation is three to four times slower. This observation was made over the entire range of atr/opsin molar ratios, including the physiologically relevant range of <1.0.

Repalmitoylation Restores the Activity of the Opsin-Atr Complex—To exclude that the effect of repalmitoylation was the result of side effects inferred by the procedure, we tried to repalmitoylate opsin by palmitoyl-CoA. The data show (Fig. 5A) that this procedure restores the original activity almost quantitatively. A qualitative control of whether the original sites were repalmitoylated is given by the data in Fig. 5B. The incorporation of $^3$H-labeled palmitoyl-CoA into native opsin is very low (lanes 1 and 2), depalmitoylated opsin incorporates a substantial amount of label (lane 3), whereas opsin repalmitoylated with unlabeled palmitoyl-CoA shows a much smaller incorporation of the label (lane 4).

pH Dependence of Gt Activation by Opsin-Atr—pH rate pro-
the termination of the opsin-atri activity by stimulating the binding of the inverse agonist, 11-cis-retinal.

The most characteristic difference between Meta II and opsin-atri comes from the photoregeneration experiment (Fig. 3). In Meta II, atr is linked to Lys by a Schiff base bond. When the chromophore is isomerized by UV light, most of the opsins react back to the ground state, as is seen in the readily observed reprotonation of the Schiff base (Fig. 3). The lack of any such signal in the case of the opsin-atri complex is evidence that light absorption fails to restore the ground state, indicating a fundamentally different chromophore/protein interaction than in Meta II.

These experiments support the hypothesis of a second binding site for atr, which may be prevented from reaching the 11-cis-retinal binding pocket in native opsin because of steric hindrance.

**Binding Sites at G, May Be Similar for Meta II and Atr—**In a recent study, the effect of synthetic peptides from the COOH-terminal stretches Gα (340–350) and Gγ (50–71)-farnesyl was investigated by several biophysical assays, identifying these two sites as part of the Gα domain to which Meta II couples in light-dependent nucleotide exchange catalysis (8). In this regard, the opsin-atri complex displays a similarity to Meta II. The peptide competition data (Fig. 1) provide evidence that opsin-atri uses the same sites for catalytic interaction with Gα as Meta II.

These analogies between the G-protein coupling mechanisms of opsin-atri and Meta II do not exclude that Meta II and/or opsin-atri uses more and other interaction sites on Gα than the ones that have been identified (37, 38). Regarding the rhodopsin/Gα interface, recent analyses argue for a complex interplay between binding sites, in that mutations at the NH2-terminal side of the fourth loop of rhodopsin affect light-dependent interactions with both Gα and Gγ-farnesyl COOH-terminal peptides. Such relationships remain to be explored for opsin-atri.

A rigorous quantitative evaluation of the peptide competition data in terms of affinities is difficult (8, 30) because of limitations of the fluorescence assay in the case of Meta II. This prevents a quantitative comparison with opsin-atri. Nevertheless, we can state that, compared with Meta II, competition for opsin-atri was enhanced with both peptides. This is qualitatively consistent with the notion that the affinity of the opsin-atri complex toward the Gα holoprotein is reduced. A similar conclusion can be reached from the shift of the alkaline branch of the pH/rate profile of Gα activation with opsin-atri compared with Meta II (Fig. 6). It reflects a lower capacity for forced protonation and thus a weaker coupling between the noncovalent retinal binding and the Gα interaction domain.

**Palmitoylation Is Essential for Atr/Opsin/G α Interaction—**The data show that palmitoylation strongly decreases the activity of opsin-atri, and palmitoylation of opsin restores the original level of Gα activation. This establishes a functional role for this modification, which was not found for Meta II, in agreement with previous studies (19, 20). We conclude that the palmitoyl thioesters at Cys and Cys are involved in binding the retinal and/or in the transmission of the agonist binding signal to the Gα interaction domain. We hypothesize that the hydrophobic interactions within the region of the

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**Fig. 5. Effect of palmitoylation of opsin on G α activation by the opsin-atri complex.** Panel A, restoration of opsin-atri-catalyzed Gα activation by autoacylation with palmitoyl-CoA as described under “Experimental Procedures.” Panel B, [3H]palmitate incorporation into opsin and dithiothreitol (DTT)-treated, depalmitoylated opsin preparations was performed as a qualitative control of the efficiency of autoacylation. Lane 1, native (palmitoylated) opsin; lane 2, same as lane 1, but after preincubation with unlabeled palmitoyl-CoA, lane 3, depalmitoylated opsin; lane 4, same as lane 3, but after preincubation with unlabeled palmitoyl-CoA. The fluorogram (exposure time 3 days) shows that [3H]palmitate is incorporated at high levels into the depalmitoylated opsin (lane 3) and that less label is incorporated if the depalmitoylated opsin is first autoacylated with unlabeled palmitoyl-CoA.

**Fig. 6. pH/rate profiles for Meta II- and opsin-atri-catalyzed G α activation.** The relative activation rates yield the “bell-shaped” profile for the photoactivated Meta II signaling state (open squares). The data were fit to a product of two titration curves as described previously (1), yielding pK5 = 5.2 and pK6 = 9. The opsin-atri complexes, palmitoylated (closed squares) and depalmitoylated (closed circles), differ strongly from Meta II and yield pK5 = 7.4 and pK7 = 7.1 for opsin and depalmitoylated opsin, respectively.
palmitoylated cysteines and the fourth loop contribute to reversible binding with atr, possibly forming part of the atr binding site. It is intriguing to note that prominent hydrophobic elements at opsin and at Gt, namely the receptor palmitoyl, Gtα-myristoyl, and Gtγ-farnesyl moieties, are involved in both receptor/Gt interaction and in membrane anchoring (39).

Physiological Implications—A physiological role for the opsin-atr complex as a storage form of atr was recently found in mice lacking the rim protein of rod outer segment discs, a member of the ABC transporter superfamily. In these retinas, dark adaptation is delayed, which was interpreted as an accumulation of the opsin-atr complex, related to impaired transport of atr and/or its adduct N-retinylidene phosphatidylethanolamine (14). The rim protein is a component of a larger signaling complex organized by glutamic acid-rich protein and localized at the disc rims, which interacts with active phosphodiesterase but not with rhodopsin or Gt (40). If it is true that atr interacts with opsin’s palmitoyl moieties, it will be of interest to analyze how it is translocated to the export machinery of the ABC transporter protein.

Conclusion—Exogenous atr activates opsin by a different mechanism than the atr in the Meta II photoprodut. The receptor structure is flexible enough to accept the chromophoric ligand in different modes of action. Even though key features of the interaction with Gt are similar, it is likely that exogenous atr uses a second binding site on bovine opsin. Palmitoylation plays a unique role in the interaction with atr and in membrane anchoring (39).

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