A 48 kDa protein arrests cGMP phosphodiesterase activation in retinal rod disk membranes

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Photolyzed rhodopsin (R*) catalyzes GTP-binding to α-transducins (Tα); Tα·GTPs then activate cGMP phosphodiesterase (PDE). PDE activation is arrested by ATP in two ways: (i) initial velocity is suppressed, and (ii) PDE velocity rapidly returns to preactivation levels (turnoff). Arrestin (a 48 kDa protein) markedly enhances turnoff while not affecting initial velocity. Arrestin in the presence of ATP achieves rapid turnoff by directly inhibiting activated PDE, as indicated by its ability to inhibit the direct activation of PDE by Tα·GMP–PNP (guanylyl-imidodiphosphate). Double reciprocal plots reveal a competition between arrestins and activated transducins for sites on PDE. Blocking R* phosphorylation blocks initial velocity suppression but does not disturb rapid turnoff. Our data suggest a 2-fold mechanism for PDE deactivation: (i) formation of Tα·GTPs is suppressed by R* phosphorylation, while (ii) activation of PDE by Tα·GTPs is competitively inhibited by arrestins when ATP is present.

Photoreceptor *Phosphodiesterase* *Transducin* *Arrestin* *Rhodopsin* *Phosphorylation*

1. INTRODUCTION

Bleaching one or more of the 10^4–10^6 copies of rhodopsin on a retinal rod disk membrane (RDM), in the presence of GTP, triggers the amplified binding of GTP to the α-subunit of the guanine nucleotide-binding protein transducin, forming Tα·GTPs [1,2]. The Tα·GTPs in turn activate cGMP phosphodiesterase (PDE) [3]. Addition of micromolar concentrations of ATP to the reaction mixture causes two changes in the kinetics of flash-induced PDE activation [4,5]. First, the initial velocity of activation is partially suppressed. Second, the time required for activation to turn off, to return to its dark velocity, is dramatically shortened.

Abbreviations: R*, photolyzed rhodopsin; Tα, α-subunit of G-protein; cGMP, cyclic guanosine-3′:5′-monophosphate; GMP-PNP, guanylyl-imidodiphosphate; DTT, dithiothreitol; Tβγ, β, and γ-subunits of G-protein; A, arrestin, 48 kDa protein, S-antigen; RK, rhodopsin kinase

We previously reported that a 48 kDa protein was involved in the ATP-dependent shortening of turnoff time in RDM preparations [6,7]. The 48 kDa protein rapidly 'arrests' PDE activation, with little or no effect on initial velocity, thus prompting us to call the 48 kDa protein arrestin [6,7]. Wilden et al. [8] have suggested that arrestin achieves turnoff by binding to bleached, phosphorylated rhodopsin, thereby deactivating it, while the PDE remains active until its activators decay through the hydrolysis of Tα·GTPs to Tα·GDPs. However, GTPase activity of transducing is a relatively slow process (20–300 s GTP^{-1}) [9,10] compared to the rapid inactivation of PDE observed in the presence of ATP (~5–15 s) [4]. We alternatively have proposed that arrestin in the presence of ATP directly inhibits the phosphodiesterase to achieve rapid turnoff [6,11].

In the present report we demonstrate that arrestin and ATP can significantly inhibit the direct activation of PDE by the stable activated form of transducin (Tα·GMP-PNP). We further show that arrestin and ATP can turn off flash-induced

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PDE activation in the presence of the nonhydrolyzable GTP analog, GMP-PNP, contrary to expectations of the Wilden et al. model [8]. Moreover, we present evidence that the initial velocity suppression by ATP results from rhodopsin phosphorylation, while rapid turnoff requires arrestin and ATP. We therefore suggest a 2-fold mechanism for the ATP-dependent quench: (i) the rate of formation of activators, $T\alpha \cdot GTPs$, is suppressed by rhodopsin phosphorylation, while (ii) activation of PDE by $T\alpha \cdot GTPs$ is competitively inhibited by arrestins in the presence of ATP [11]. We have no evidence that rhodopsin phosphorylation is required for the arresting action of the 48 kDa protein on PDE.

2. MATERIALS AND METHODS

2.1. *Materials*

Fresh bovine eyes were purchased from MOPAC, Souderton, PA. [γ-^{32}P]ATP was obtained from ICN (2000–5000 Ci/mmol). cGMP, Mops and salts were purchased from Sigma; ATP, GTP, GMP–PNP and DTT were obtained from Boehringer Mannheim.

2.2. *Preparation of rod disk membranes*

RDM were prepared by a standard method [12], with all procedures carried out under infrared illumination with the aid of image converters.

2.3. *Purified proteins*

Arrestin was purified from RDM preparations by FPLC (Pharmacia) or by low pressure liquid chromatography. A hypotonic extract (10 mM Tris, 1 mM DTT, pH 7.5) of bovine RDM in the dark was applied to a Mono Q anion-exchange column, and eluted by a linear salt gradient (0–500 mM NaCl, 50 mM Tris, 1 mM DTT, pH 7.5). Arrestin eluted at $\simeq 50\%$ on the salt gradient. Such preparations were typically 95% pure and gave similar results to those further purified to homogeneity on a TSK molecular sieve column.

$T\alpha$-enriched transducin in the long-term activated form ($T\alpha \cdot GMP\text{-}PNP$) was extracted from bleached, washed RDM with GMP-PNP at moderate ionic strength according to the procedures of Kühn and Wilden [13]. The purified protein contained at least 85% $T\alpha$, with $T\beta\gamma$ as the major impurity. $T\alpha \cdot GMP\text{-}PNP$ was separated from free nucleotide on a Sephadex G-25 column (Pharmacia, PD-10).

2.4. *Rhodopsin phosphorylation*

Rhodopsin phosphorylation was carried out in cGMP phosphodiesterase assay mixtures. High specific activity [γ-^{32}P]ATP was added 15 s prior to a light flash calibrated to bleach a desired fraction of rhodopsin. Reactions were stopped 30 s after the flash with an equal volume of electrophoresis buffer containing 4% SDS. The samples were electrophoresed in 15% SDS-polyacrylamide gels [10]. After staining and destaining the rhodopsin bands were cut out and digested in Protosol (NEN) diluted 1:10 with Ultrafluor (National Diagnostics).

2.5. *PDE assay*

PDE velocity was monitored by a real-time pH assay using a modification of the method of Liebman and Evanczuk [14]. The reaction mixture contained 4 μM rhodopsin and 5 mM cGMP in Mops buffer with additional nucleotides as indicated. Samples (0.5 or 0.75 ml) were incubated in a thermostatically regulated cuvette with magnetic stirrer. A miniature pH electrode (Beetrode, WPI) and KCl-filled Ag/AgCl reference were connected to a high input impedance electrometer (WPI), whose output was fed to a chart recorder and stored on FM tape. The reaction was normally started by bleaching a calibrated fraction of rhodopsin with a photographic strobe (1 ms duration). The flash was spectrally shaped by a Wratten 57 filter and attenuated by calibrated neutral density filters. Experiments were performed in complete darkness with the aid of IR image converters.

2.6. *Protein assays*

Protein concentration was determined by Bradford's assay [15], with bovine serum albumin used as a standard. Rhodopsin concentration was determined from its absorbance at 502 nm in 1% Ammonyx LO.

3. RESULTS

3.1. *Arrestin shortens turnoff time in RDM*

Fig.1. demonstrates the essentially exclusive effect of arrestin on turnoff time in bovine RDM preparations. Flash intensity was increased until

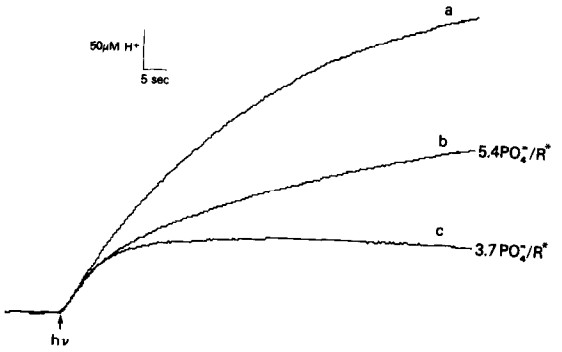

Fig.1. Arrestin shortens turnoff time in the presence of ATP in RDM, with no effect on initial velocity. Stoichiometric proton release with cyclic GMP hydrolysis following a flash ($h\nu$) of 1 ms duration that bleaches a 2.9×10^{-4} fraction of the rhodopsin in the presence of 250 μM GTP cofactor (a-c). Curves b and c contained 500 μM ATP added prior to the flash. Curve c contained 14 μg purified bovine arrestin prepared according to the procedures outlined in section 2. All traces from separate disk membrane samples of the same preparation contained 4 μM rhodopsin, 5 mM cyclic GMP, 2 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 20 mM Mops, pH 8.0, with $T = 25°C$ and reaction volume = 0.5 ml. Membranes were from fresh bovine material for this and subsequent figures. Linear dark rates have been subtracted from the records. Rhodopsin phosphorylation performed on identical preparations at identical bleach levels in the presence of 50 μM ATP containing about 30 μCi of [γ-^{32}P]-ATP added 15 s prior to the flash and quenched in SDS-containing electrophoresis buffer 30 s after flash. The samples were electrophoresed according to [10]; the rhodopsin bands were cut out and counted for incorporated $^{32}P_i$ in Triton/toluene scintillation fluid. Reaction mixtures that were not bleached under similar conditions served as dark controls. Incorporated $PO_4^=/R^*$ in b and c are each means of light-dark differences from 5 light and 5 dark samples.

the preparation failed to show rapid turnoff in the presence of ATP and GTP (curve b). The addition of purified bovine arrestin (gel shown in fig.4C) to an RDM preparation at this bleach level (2.9×10^{-4} fraction bleached) significantly shortens turnoff time, i.e., the time required for PDE activation to return to its dark velocity, while having no effect on initial velocity of activation (curve c). To determine whether the quenching effects of arrestin are in some way mediated by rhodopsin phosphorylation we added high specific activity [γ-^{32}P]ATP to parallel samples of RDM, with and without added arrestin, and measured rhodopsin phosphorylation at the same bleach level used in the pH assay. As shown, the shortening of turnoff time by arrestin is achieved without increase in the magnitude of rhodopsin phosphorylation. Consistent with the findings of Pfister et al. [16], we find arrestin isolated by anion-exchange chromatography to be devoid of rhodopsin kinase activity. In addition, the effect of arrestin is not a nonspecific protein effect as 10-fold higher concentrations of ovalbumin do not influence turnoff. Similar effects of arrestin on turnoff in RDM preparations have been recently reported by others [8].

3.2. *Decay of Tα · GTP to Tα · GDP is not required for deactivation of PDE by arrestin*

Wilden et al. [8] have proposed that the sole effect of arrestin is to deactivate R* by binding to phosphorylated R*, while PDE ultimately deactivates through the hydrolysis of Tα · GTPs to Tα · GDPs. According to their model, GTP hydrolysis is an absolute requirement for turnoff of PDE activation, and it should therefore be impossible to turn off a reaction in which a nonhydrolyzable GTP analog, GMP-PNP, is substituted for GTP.

Fig.2 shows that flash-induced PDE activation in the presence of low concentrations of GMP-

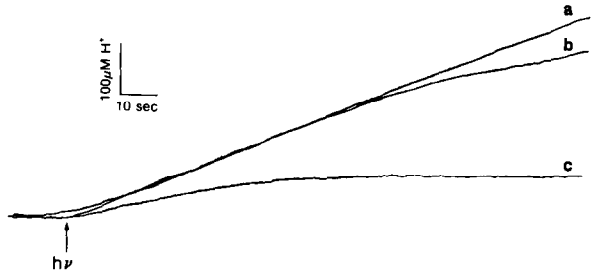

Fig.2. Arrestins turns off PDE activation in the absence of GTP hydrolysis. pH assay of cyclic GMP hydrolysis in the presence of 10 μM GMP-PNP, a nonhydrolyzable GTP analog, following a flash that bleached a 3.0×10^{-5} fraction of the rhodopsin (a-c). Curves b and c contained 250 μM ATP added prior to the flash. Curve c contained 17 μg purified bovine arrestin added prior to the light flash. All traces from separate RDM samples of the same preparation contained 4 μM rhodopsin, 5 mM cyclic GMP, 2 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 20 mM Mops, pH 8.0, with $T = 30°C$ and reaction volume = 0.75 ml. Linear dark rates have been subtracted from the records.

PNP (10 μM) can be turned off by arrestin and ATP. In curve a, a flash bleach causes essentially straight-line activation in the presence of 10 μM GMP-PNP, as the activators formed by the flash (Tα · GMP-PNPs) cannot be hydrolyzed. In curve b, 250 μM ATP does little to such activation in RDM, but in curve c the addition of 17 μg purified bovine arrestin and ATP causes activation to return to its dark velocity, thus turning off PDE activation.

The experiment in fig.2 proves that the capping of phosphorylated R* by arrestin cannot be its only inhibitory action, and is consistent with, but alone does not prove, a model in which arrestin in the presence of ATP directly inhibits activated PDE. The following experiments were designed to test this model.

3.3. *Arrestin and ATP directly inhibit activated PDE*

PDE was activated directly in dark RDM by adding the stable activated form of transducin (Tα · GMP-PNP), thus bypassing the normal activation pathway through R*. In fig.3, the addition of Tα · GMP-PNP at time A, to a dark preparation containing 50 μM ATP, causes PDE activation, indicated by the upward slope. A subsequent flash bleach serves two functions. First, the flash, which fails to increase PDE activation above that caused by the Tα · GMP-PNP, demonstrates that there is no free GMP-PNP in the cuvette, and therefore that PDE is not being activated through R* and endogenous transducin. The PDE, then, is being directly activated by the exogenously applied activators, Tα · GMP-PNPs. Second, the flash forms R*s required for the activation of arrestin, which is added at time B. The addition of arrestin, after a slight pH jump, depresses PDE velocity by approximately 80%. Arrestin and ATP, therefore, directly inhibit activated PDE. Interaction of arrestin with R* is required for its inhibitory action on PDE, as arrestin is without effect in the absence of R* (not shown).

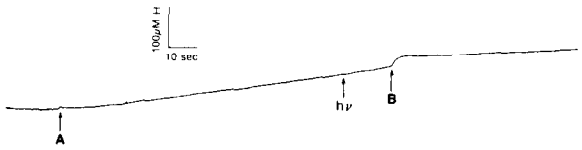

Fig.3. Evidence that arrestin in the presence of ATP directly inhibits activated PDE. pH record from reaction cuvette containing 5 mM cyclic GMP and 50 μM ATP added prior to time A. At A, 3 μg of the stable PDE activator Tα · GMP-PNP was added to the cuvette. Light flash ($h\nu$) that bleached a 3.0×10^{-5} fraction of rhodopsin failed to increase activation above that caused by Tα · GMP-PNP, indicating that PDE is not activated through R* and endogenous transducin, but is being activated by exogenously applied Tα · GMP-PNPs. At B, the 25 μg purified arrestin added to the cuvette depresses PDE velocity by ~80%. Reaction buffer, rhodopsin concentration, temperature and volume are the same as in fig.2.

3.4. *Arrestins and activated transducins compete for sites on PDE*

To delineate the mechanism of direct inhibition of PDE by arrestin we have repeated experiments similar to those shown in fig.3 over a range of Tα · GMP-PNP concentrations in the presence and absence of arrestin and/or ATP. Gels of the purified proteins used, Tα · GMP-PNP and arrestin, are shown in fig.4, panel C, and representative pH records shown in panel A. In this series of experiments arrestin and/or ATP were added prior to the light flash, and PDE velocity measured subsequent to the addition of varied concentrations of Tα · GMP-PNP, added at time A (fig.4A). PDE velocity was measured along the straight-line portion of activation subsequent to Tα-GMP-PNP addition. Control experiments revealed, as in fig.3, that the system was unable to activate through R* and endogenous transducin because it lacks free guanosine nucleoside triphosphate; PDE is thus being directly activated by added Tα · GMP-PNPs.

Double reciprocal plots of the data are shown in fig.4B. The addition of arrestin and ATP (solid squares) significantly increases the slope, indicating inhibition of the direct activation of PDE by Tα · GMP-PNP. ATP in the absence of arrestin (open circle) has no effect on PDE velocity, and arrestin in the absence of ATP (solid circle) is also without effect. Direct inhibition of activated PDE, then, requires arrestin and ATP.

Linear regression lines through the data points intersect on the $1/v$ axis indicating competitive inhibition, i.e., activated transducins and arrestins compete for sites on PDE. Wilden et al. [8] failed to observe a direct inhibitory effect of arrestin on PDE using 3-fold higher concentrations of activators than those at which we observed inhibition. This is not surprising, as the definition of

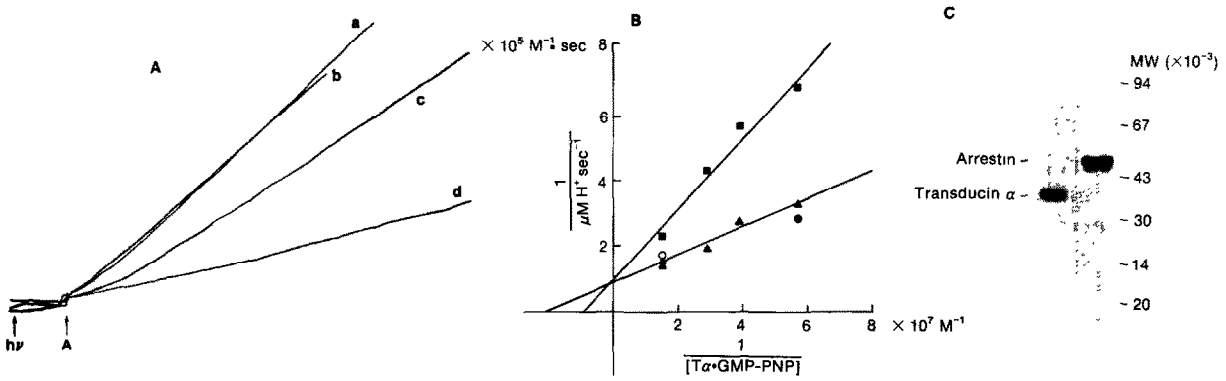

Fig.4. Activation of PDE by Tα · PNPs is competitively inhibited by arrestins in the presence of ATP. Representative pH records in panel A, double reciprocal plot of data in panel B, gels of purified proteins in panel C. (A) 10 μg purified arrestin and/or 100 μM ATP added, when indicated, prior to a flash that bleached a 1.6×10^{-5} fraction of rhodopsin. Curves: (a) no prior additions, 2 μg Tα · GMP-PNP injected into cuvette at A; (b) 100 μM ATP added prior to flash, 2 μg Tα · GMP-PNP added at A; (c) no prior additions, 1 μg Tα · GMP-PNP added at A; (d) 10 μg arrestin and 100 μM ATP added prior to flash, 1 μg Tα · GMP-PNP added at A. Reaction buffer, rhodopsin concentration, temperature and volume as in fig.2. (B) Arrestin (10 μg) + 100 μM ATP + Tα · GMP-PNP (■); Tα · GMP-PNP (▲); Tα · GMP-PNP + arrestin (10 μg) (●); Tα · GMP-PNP + 100 μM ATP (○). (C7 SDS-PAGE of purified proteins used in this figure, panels A and B, and in figs 1–3. Left lane, enriched Tα · GMP-PNP; right lane, purified arrestin prepared as in section 2.

competitive inhibition predicts, as do our data, that such high substrate concentrations should overcome the inhibitor, thus explaining their negative result.

If we assume linear competitive inhibition, we can compute an inhibitor constant, K_I, from the data, and arrive at a value of 193 nM. This translates into approx. 1 arrestin per 20 rhodopsins, a highly selective inhibitory effect comparable to inhibition by the γ-subunit of PDE on light activation recently reported by Sitaramayya et al. [17].

3.5. *Rhodopsin phosphorylation accounts for the initial velocity component of quench*

To explore the role of rhodopsin phosphorylation in the ATP-dependent quench, we have employed monoclonal anti-rhodopsin antibodies against phosphorylation sites on the C-terminus of rhodopsin. The monoclonal antibodies employed were those prepared and characterized by Molday and MacKenzie [18]. At GTP concentrations below the K_m for rhodopsin phosphorylation by GTP [19] (Fig.5 curves a and b), monoclonal 1D4

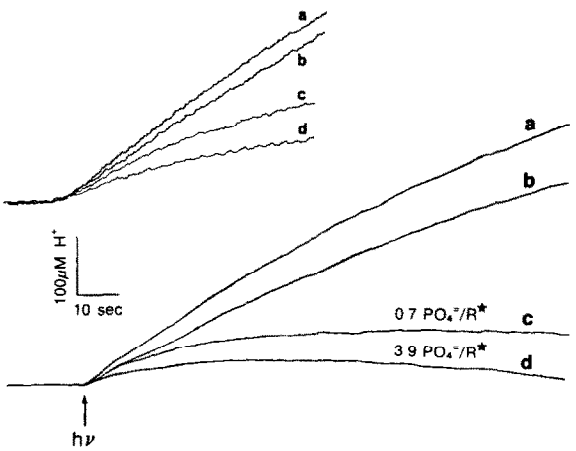

Fig.5. Evidence that rhodopsin phosphorylation causes initial velocity suppression, but is not required for rapid turnoff by arrestin and ATP. Proton release with cyclic GMP hydrolysis following a flash that bleached a 1.5×10^{-5} fraction of rhodopsin in the presence of 50 μM GTP cofactor (curves a–d). Curves c and d contained 100 μM ATP added prior to the light flash. Curves a and c from RDM samples incubated on ice for 1 h with 4 μM 1D4 monoclonal antibody. Rhodopsin phosphorylation performed as in fig.1, with $PO_4^=/R^*$ in c and d each representing mean light-dark differences from 5 light and 5 dark samples. Reaction buffer, rhodopsin concentration, temperature and volume as in fig.2. Inset shows initial velocities of activation at increased gain. Calibration bars pertain to lower pH records only. Linear dark rates have been substracted from the records.

only slightly increases activation. ATP phosphorylates R* (curve d), as measured at 30 s under identical weak bleach conditions to 3.9 $PO_4^{=}$/R*, and rapid turnoff is observed in the RDM preparation. Monoclonal 1D4 blocks phosphorylation to 0.7 $PO_4^{=}$/R*, meaning that on average 30% of the R*s have no phosphates on them. Blocking phosphorylation (curve c) increases initial velocity of activation; but in the presence of endogenous arrestin, the reaction still turns off. Therefore, phosphorylation of R* accounts for the initial velocity suppression component of the quench, but is not a necessary condition for rapid turnoff of PDE by arrestin and ATP. Similar results were also obtained with another monoclonal antibody, 3A6. A more detailed account of the effects of blocking rhodopsin phosphorylation on the quench will appear elsewhere.

4. DISCUSSION

The data lead us to suggest the following model of PDE activation and deactivation:

	1. $R^0 + h\nu$	$\xrightarrow{}$	R**	
Activation	2. $T\alpha^0 \cdot GDP + GTP$	$\xrightarrow{R^{**}}$	$T\alpha^* \cdot GTP$	
	3. $T\alpha^* \cdot GTP + PDE^0$	$\longrightarrow$	$GTP \cdot T\alpha^* \cdot PDE^*$	c G 5′-GMP + H^+
	4. $A^0 + ATP$	$\xrightarrow{R^{**}}$	A*	
	5. R** + nATP	$\xrightarrow{RK}$	R*Pn + nADP	
Deactivation	6. $A^* + GTP \cdot T\alpha^* \cdot PDE^*$	$\longrightarrow$	$A^* \cdot PDE^0 + T\alpha \cdot GTP$	
		and		
	7. $A^* + PDE^0$	$\longrightarrow$	$A^* \cdot PDE^0$	

R**, most likely metarhodopsin II [20], formed by the bleaching of rhodopsin, catalyzes the exchange of GTP for bound GDP on the α-subunit of transducin [1,2]. $T\alpha^* \cdot GTP$s formed by this reaction bind to an inactive PDE molecule, PDE^0, thus activating it [3]. The active diesterase, PDE*, hydrolyzes cyclic GMP until it is deactivated by the action of A [11]. R** also catalyzes the formation of a deactivating form of arrestin. A*, which competes for sites occupied by $T\alpha^* \cdot GTP$s on PDE*, deactivating the active diesterases to yield PDE°. Similarly, A*s bind to inactive diesterases, PDE°, to prevent their activation by $T\alpha \cdot GTP$s. Concomitant with the deactivation of PDE by A*s, R** is being phosphorylated by RK and ATP. The phosphorylation of R** partially deactivates it [8,21], forming R*Pn, which has a significantly diminished capacity to catalyze the formation of $T\alpha^* \cdot GTP$s, but which is still competent to catalyze the conversion of arrestins to their deactivating form, A*s.

The system would need to recover from deactivation through a number of processes, some of which remain to be worked out. First, the $T\alpha^* \cdot GTP$s hydrolyze to the inactive form of transducin, $T\alpha^\circ \cdot GDP$s [9,10]. Second, R*Pn dephosphorylates and passes to an inactive intermediate in the bleaching sequence, R°. Third, A* dissociates from PDE, and returns to its inactive form A° through reactions yet to be determined.

The reactions involved in the R**-catalyzed formation of deactivating arrestins are currently being investigated. We [22], and more recently others [23], have shown that arrestin is an adenine nucleotide-binding protein, labeling with the photoaffinity ligand 8-azido[α-^{32}P]ATP. Moreover, partial sequencing of arrestin has revealed homologies with other purine nucleotide-binding proteins [24]. Furthermore, arrestin is involved in effecting changes in unbound cytosol ATP and ADP [6,25]. We believe that the deactivation of PDE by arrestin is intimately related to the presence and form of a bound adenine nucleotide on arrestin; further work will reveal the relevant binding reactions involved.

The present model explains features of PDE activation and deactivation which were hitherto unexplained. First, the reported weak binding of activators, $T\alpha^* \cdot GTP$s, to PDE [17] would be reasonable if a deactivating molecule, A*, is to compete effectively with activator molecules on PDE. Second, the model is consistent with

previously reported low values for GTPase velocity [9,10], as GTP hydrolysis is involved in the slow recovery of the system, and not in its rapid deactivation as proposed by others [8]. This further implies that arrestin is functionally most important at higher bleach levels at which the GTPase is least able to keep up with the amplified production of $T\alpha \cdot GTPs$. Third, the only requirement for deactivation of the system by arrestin according to Wilden et al. [8] is phosphorylation of R*. However, GTP has also been shown to phosphorylate R*; yet in the presence of concentrations of GTP alone which exceed its K_m for R* phosphorylation [19] (e.g., fig.1a), the system fails to show rapid turnoff of PDE activation. We propose that the formation of A*s, which directly inhibit activated PDEs, is an ATP-specific reaction, consistent with the characterization of arrestin as an adenine nucleotide-binding protein [22].

The present work which demonstrates that arrestin achieves rapid turnoff by directly inhibiting the disk complement of PDEs does not, however, rule out a role for arrestin in deactivating R* as proposed by Wilden et al. [8]. Further work will be necessary to resolve the relative contributions of the two proposed roles for arrestin in achieving quench, thus allowing a theoretical model to be formulated which can deal with the kinetic subtleties of light-induced PDE activity.

The hormonally-activated adenylate cyclase shares similarities with the cGMP phosphodiesterase in its activation, and turns off through a process termed desensitization [26]. It will be interesting to see whether desensitization involves a protein homologous to arrestin, or whether arrestin is a unique evolutionary adaptation to the needs of phototransduction.

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