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Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments†

(photoreceptor/GTP-binding protein/transducin/retinal S-antigen/arrestin)

U. WILDEN, S. W. HALL, AND H. KÜHN

Institut für Neurobiologie, der Kernforschungsanlage Jülich GmbH, 5170 Jülich, Federal Republic of Germany

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ABSTRACT **Each photoexcited rhodopsin (R*) molecule catalyzes binding of GTP to many copies of the guanine nucleotide-binding protein transducin, which, in its GTP-binding form, then activates cGMP phosphodiesterase (PDEase). Subsequent deactivation of this light-activated enzyme cascade involves hydrolysis of the GTP bound to transducin, as well as decay of the activating capacity of R*. We report here that deactivation of PDEase in rod outer segment suspensions is highly enhanced by addition of ATP and purified 48-kDa protein, which is an intrinsic rod outer segment protein that is soluble in the dark but binds to photolyzed rhodopsin that has been phosphorylated by rhodopsin kinase and ATP [Kühn, H., Hall, S. W. & Wilden, U. (1984) *FEBS Lett.* 176, 473–478]. To analyze the mechanism by which ATP and 48-kDa protein deactivate PDEase, we used an ATP-free system consisting of thoroughly washed disk membranes, whose rhodopsin had been previously phosphorylated and chromophore-regenerated, and to which purified PDEase and transducin were reassociated. Such phosphorylated membranes exhibited a significantly lower (by a factor ≤5) light-induced PDEase-activating capacity than unphosphorylated controls. Addition of purified 48-kDa protein to phosphorylated membranes further suppressed their PDEase-activating capacity; suppression could be as high as 98% (as compared to unphosphorylated membranes), depending on the amount of 48-kDa protein and the flash intensity. Addition of ATP had little further effect. In contrast, PDEase activation or deactivation with unphosphorylated control membranes was not influenced by 48-kDa protein, even in the presence of ATP, provided rhodopsin kinase was absent. Our data suggest that 48-kDa protein binds to phosphorylated R* and thereby quenches its capacity to activate transducin and PDEase.**

Absorption of light converts rhodopsin into an active form, "photoexcited rhodopsin" (R*), that specifically interacts with three proteins of the rod cell (1). First, a guanine nucleotide-binding protein, transducin,‡ transiently binds to R* (4). This enables the exchange of GTP for previously bound GDP (3) on the α subunit of transducin (T_α), which then, in its GTP-binding form (T_α-GTP), dissociates from R* (4, 5) and activates a cGMP phosphodiesterase (PDEase) (6). One R* can, during its lifetime, catalyze nucleotide exchange on several hundred transducin molecules (3, 6) and, therefore, lead to the activation of several hundred PDEase molecules (7, 8).

Second, a soluble protein kinase, specific for photobleached rhodopsin, binds to R* (9) and phosphorylates it at multiple serine and threonine residues (10, 11). Third, another soluble protein, "48-kDa protein," also binds to bleached disk membranes (9) particularly well if their rhodopsin is phosphorylated (12).

Phosphorylation of rhodopsin has been proposed (13, 14) to function as a "stop" signal, terminating the active state of R* faster than the spontaneous slow decay of the active photoproduct (metarhodopsin II; see refs. 15 and 16) would occur. Specifically, Liebman and Pugh (14) showed that addition of ATP to rod outer segment (ROS) fragments leads to highly accelerated deactivation of PDEase at low bleaching levels, and that the K_m for ATP of this PDEase-quenching effect resembles that of rhodopsin kinase. Miller and Dratz (17) have shown that proteolytic removal of most of the phosphorylation sites from rhodopsin abolishes the ATP-mediated deactivation of PDEase.

We show here that, in addition to ATP, 48-kDa protein is also required to effectively deactivate PDEase. Since ROS are complex organelles in which ATP has many effects besides phosphorylation of rhodopsin (e.g., ref. 18), we used a better defined system of previously phosphorylated rhodopsin membranes to which purified PDEase, transducin, and 48-kDa protein were added. Our results indicate that 48-kDa protein binds to phosphorylated R* and thereby blocks the capacity of R* to activate transducin and PDEase.

MATERIALS AND METHODS

Membrane Preparations. ROS were purified from fresh bovine eyes as described (11). They were stored frozen under argon at −70°C.

Phosphorylated ROS membranes (P-disks) were prepared as described (12). Briefly, ROS suspended in 100 mM phosphate buffer were illuminated in the presence of 3 mM [γ-^{32}P]ATP (New England Nuclear) and 1.5 mM $MgCl_2$ for 3 hr at 30°C, regenerated in the dark with excess 11-*cis*-retinal, and washed extensively with isotonic and hypotonic buffers. The final P-disks were resuspended in PDEase assay buffer (see below) and stored frozen at −70°C in aliquots. Phosphorylation yields were 5.5–6.0 phosphates bound per average rhodopsin, and regeneration yields were 98–102%. Analysis of detergent-solubilized aliquots on ECTEOLA-cellulose columns (11) revealed that 2% (±1%) of the rhodopsin always remained unphosphorylated, whereas the rest was

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Abbreviations: ROS, rod outer segments; P-disks, phosphorylated and washed ROS membranes; R*, photoexcited rhodopsin; T_α, α subunit of transducin; GTP[γ-S], guanosine 5′-[γ-thio]triphosphate; PDEase, cGMP phosphodiesterase.

†Part of this work has been presented at a Roussel-Uclaf conference on "Mechanism of Vision" (Paris, November 22–23, 1984), and at the 36th Mosbacher Kolloquium on "Selected Topics of Neurobiochemistry" (Mosbach F.R.G., April 18–20, 1985).

‡This guanine nucleotide-binding protein has been termed GTPase (2), GTP-binding protein, G-protein, and transducin (3). We adopt the name "transducin" in order to distinguish it from other "G-proteins" (e.g., those of the hormonal system).

phosphorylated to various levels. Nonphosphorylated control disks were prepared similarly but in the absence of ATP. Before PDEase assays, the membranes were incubated for 2–3 hr at 30°C with 1 mM NADPH; this treatment helped suppress dark activity but did not significantly influence light-induced PDEase activity (W. J. de Grip, personal communication).

Purified Proteins. Transducin in the GDP-binding form ($T_{\alpha\beta\gamma}$–GDP) was purified as described (5); excess GTP and GDP were removed by gel filtration on a Sephadex G-25 column. T_α enriched in the long-term activated form (T_α–GTP[γ-S]), containing about 85% T_α and 15% $T_{\beta\gamma}$, was obtained similarly except that transducin was extracted from the bleached, washed ROS membranes with guanosine 5'-[γ-thio]triphosphate at moderate ionic strength (15). Phosphodiesterase was obtained as a by-product during transducin purification (5) and was 85–90% pure.

The 48-kDa protein, which is identical with retinal S-antigen (19), was purified from whole-retina extracts by a published procedure for S-antigen purification (20). It was usually about 95% pure. Some preparations further purified to homogeneity by FPLC (21) gave the same results as the normally used, 95% pure preparations. All of the purified proteins were frozen in liquid N_2 as small aliquots and were stored at −70°C.

PDEase Activity. This was monitored through the pH changes associated with cGMP hydrolysis (7). Samples (1.4 ml) were incubated in a cuvette thermostatically regulated at 30°C and equipped with a mechanical stirrer and with a pH electrode (Amagruss) connected to a recorder. Changes in H^+ concentration were calibrated by adding known amounts of HCl to control samples. The assay mixture normally contained 150 mM KCl, 10 mM Hepes (pH 7.9), 2 mM $MgCl_2$, 1 mM dithiothreitol, 2–4 mM cGMP, 0.1 mM GTP, ≈0.07 mM NADPH, and proteins and other additions as specified in each case. Samples were preincubated in the dark for 2–3 min. The reaction was normally started by bleaching a calibrated amount of rhodopsin with a photographic flash (100-μsec duration) filtered through a green filter (500 nm) and attenuated with neutral density filters. Experiments were performed either in complete darkness or in very dim, diffuse red light.

Protein Assays. Protein concentration was determined by Bradford's assay (22) and by densitometry of Coomassie blue-stained gels (the two methods gave similar results); bovine serum albumin was used as standard. Rhodopsin concentration was determined from its light-sensitive absorbance at 500 nm (11).

RESULTS AND DISCUSSION

Deactivation of PDEase in ROS Membranes. The light-induced activity of PDEase in ROS membranes is lower and decays faster in the presence of ATP than in its absence (compare traces c and a in Fig. 1*A*), in agreement with previous reports (14, 17). This quenching effect of ATP is strongly enhanced by the addition of 48-kDa protein (Fig. 1, traces d, e, h–m). This enhancement is particularly obvious at saturating light levels (Fig. 1*B*), where ATP alone has only a very limited quenching effect (trace g) and where addition of increasing amounts of 48-kDa protein progressively quenches the PDEase (traces h–m). At lower, subsaturating light levels, which are normally used for PDEase assays (7, 8, 14), the amount of intrinsic 48-kDa protein present in the ROS preparations seems to be largely sufficient to exert its quenching effect (Fig. 1*A*). (From densitometric scans of $NaDodSO_4$/polyacrylamide gels, we estimate the amount of 48-kDa protein in our broken ROS preparations to be 0.5–1% of the rhodopsin content.) The 48-kDa protein seems to require ATP to exert its quenching effect; addition of 48-kDa protein in the absence of ATP normally has no effect, but sometimes has a small effect (Fig. 1*A*, trace b), on PDEase activity.

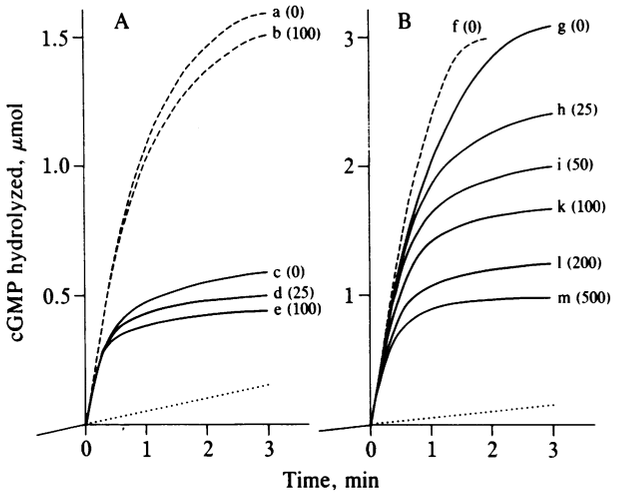

FIG. 1. ATP and 48-kDa protein quench light-induced cGMP hydrolysis in ROS membranes. Broken lines, no ATP added; solid lines, 500 μM ATP added 2–3 min before flash; dotted lines, basal (dark) activities extrapolated. Activity traces for the individual samples are lettered consecutively; numbers in parentheses denote the amounts (μg) of 48-kDa protein added 2–3 min before flash. All samples contained 6 μM rhodopsin (ROS), 100 μM GTP, and 2.2 mM cGMP. In samples f and g, all the cGMP was consumed. The fraction of rhodopsin bleached by the flash at time zero was 2×10^{-5} (*A*) or 4×10^{-4} (*B*). Note the different scales for *A* and *B*.

The results of Fig. 1 are consistent with, but do not prove, the following model. The major effect of ATP is presumably to phosphorylate R*. Since 48-kDa protein binds to phosphorylated R* and competes with transducin for binding (12), we propose that 48-kDa protein thereby inhibits the interaction between phosphorylated R* and transducin, thus preventing further activation of transducin and PDEase.

To exclude other possible reactions of ATP which occur in the complex ROS system (18) besides R* phosphorylation, we conducted the following experiments in the absence of ATP, by reassociating purified PDEase and transducin to previously phosphorylated, regenerated disk membranes (P-disks) and comparing their activating properties with those of similarly prepared but unphosphorylated control membranes.

Reassociated System. Fig. 2 shows that PDEase, reassociated, together with a molar excess of transducin, to either control disks (broken lines) or phosphorylated disks (solid lines), can be activated by light on both types of membranes. At the highest flash intensity used, V_{max} of the PDEase reassociated to control disks (Fig. 2, trace a) corresponds to a cGMP turnover number of 2200 sec^{-1}. This compares well with published maximum turnover numbers for trypsin-activated PDEase (2000 sec^{-1}, ref. 23) and indicates that purification and reassociation of the peripheral enzymes did not significantly alter their activity.

Phosphorylated disks, when flash-illuminated at medium or low light intensity, activate the PDEase to a significantly lesser extent than control disks (compare traces h with k, and q with s, in Fig. 2). Only at the highest light intensity used, did P-disks give nearly the same maximal activation as control disks (Fig. 2*A*, traces b and a); but in this case, the proportion of unphosphorylated rhodopsin that is present in the P-disk preparation (2%, see *Materials and Methods*) almost certainly contributed to the activation and might even fully account for it.

Increasing amounts of 48-kDa protein, added to P-disks, progressively inhibit PDEase activation at all light intensities

used (Fig. 2, traces c–g, l–p, and t–v). Since 40 μg of added 48-kDa protein is approximately equimolar to the amount of transducin present in each mixture, it is obvious that a relatively large molar excess of 48-kDa protein is necessary to effectively quench the PDEase, particularly at high flash intensities. At lower intensities (Fig. 2 *B* and *C*), less 48-kDa protein is required for PDEase quenching. Addition of 48-kDa protein to control disks had no or only little effect on PDEase activity (Fig. 2, traces i and r).

Comparison of the results of Figs. 1 and 2 shows that 48-kDa protein quenches the PDEase only under conditions in which R* is phosphorylated; either R* becomes phosphorylated during the PDEase assay by the ROS-intrinsic kinase and added ATP (Fig. 1, solid lines) or it had been phosphorylated prior to the PDE assay (Fig. 2, solid lines). This strongly supports our proposal that binding of 48-kDa protein to phosphorylated R* inhibits the capacity of R* to activate transducin and therefore PDEase.

As previously reported (14, 17, 24), ATP has two kinetically distinct effects on PDEase activity in ROS: it quenches the initial velocity, and it shortens the time required to turn off PDEase activity. In the ROS membrane preparation represented in Fig. 1, the effect of ATP and 48-kDa protein on PDEase initial velocity is relatively small, particularly at a high bleaching level (Fig. 1*B*), so that the effect on turn-off rate predominates. This is presumably due to the fact that R* is unphosphorylated initially and becomes phosphorylated only slowly during the PDEase assay. Separate phosphorylation experiments have shown that these ROS preparations contain rather low kinase activity (data not shown). On the other hand, if the rhodopsin has been phosphorylated and regenerated prior to the PDEase assay, as is the case with P-disks, then the initial rate of PDEase is strongly affected by phosphorylation and 48-kDa protein (Fig. 2).

The effect of rhodopsin phosphorylation and of addition of 48-kDa protein on the initial rate of light-activated PDEase is shown in Fig. 3 for a wide range of light intensities. Half-maximal activation of PDEase reassociated to control disks (upper curve) occurs when the fraction of rhodopsin bleached $= 5 \times 10^{-5}$, in exact agreement with data reported for bovine ROS (8). Phosphorylated disks (middle curve) have a reduced activating capacity, one-third to one-fifth that of control disks at medium and low light intensities. Addition of 48-kDa protein, in 10-fold molar excess over transducin (lower curve), further reduces the initial velocity, by a factor of 6–8, at medium and low light intensities. The combined action of phosphorylation and 48-kDa protein, therefore, can lead to efficient suppression of PDEase activation (98% suppression at 2×10^{-5} fraction bleached); at bleaching levels $<2 \times 10^{-5}$, light-induced PDEase activity is no longer measurable.

Phosphorylation of R* Is Required for 48-kDa Protein to Quench PDEase. Addition of 48-kDa protein to unphosphorylated control disks in the absence of ATP did not significantly quench PDEase activation (not shown in Fig. 3; but see traces i and r in Fig. 2). On the other hand, addition of 48-kDa protein plus ATP to control disks resulted in variable but sometimes significant quenching of PDEase activation. To check whether or not this was due to rhodopsin phosphorylation, we assayed the different protein preparations for the presence of rhodopsin kinase (9). We found that not only the control disks but also the semipurified PDE and even the purified transducin (see Fig. 4*B*) sometimes contained considerable activities of residual rhodopsin kinase. Only the 48-kDa protein preparations were always kinase-free, in agreement with a recent report (19).

When preparations completely free of kinase [FPLC-purified PDEase (25), hexylamine agarose-purified transduc-

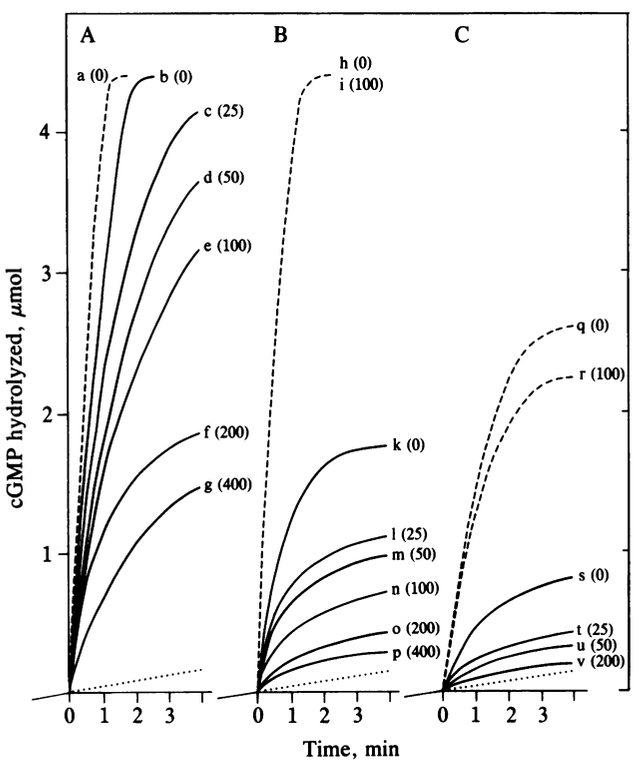

FIG. 2. PDEase activity measured with purified enzymes reassociated to either control disks (broken lines), or phosphorylated disks (solid lines after time zero). Each sample contained 5 nmol of rhodopsin, 0.75 nmol of transducin, and 0.035 nmol of PDEase in a final volume of 1.4 ml. Initial cGMP concentration was 3.3 mM. Each number in parentheses denotes the amount (μg) of 48-kDa protein added to the corresponding sample. For polypeptide composition of the purified proteins see Fig. 4*B*. The reaction was started by a flash bleaching 10^{-2} (*A*), 2×10^{-4} (*B*), or 4×10^{-5} (*C*) of rhodopsin. At maximal activity (traces a, b, h, and i), all of the cGMP was consumed. No ATP was added to any of the samples.

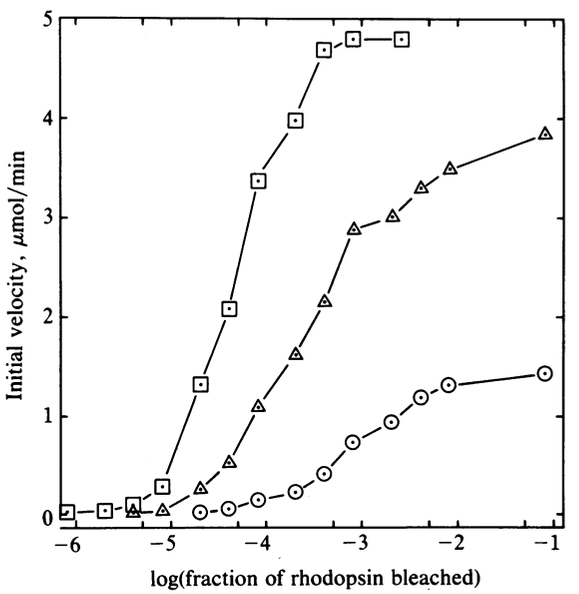

FIG. 3. Light titration of PDEase initial velocity in three different preparations. Purified PDEase and transducin were reassociated with either control disks (□), or phosphorylated disks (△), or phosphorylated disks plus 48-kDa protein (○; 400 μg per sample, corresponding to a 10-fold molar excess of 48-kDa protein over transducin). Initial cGMP concentration was 4 mM. For other concentrations and conditions, see legend to Fig. 2. Initial velocity of PDEase was determined from the slope at early times (5–30 sec) of PDEase activity traces like those shown in Fig. 2. Dark activities were subtracted.

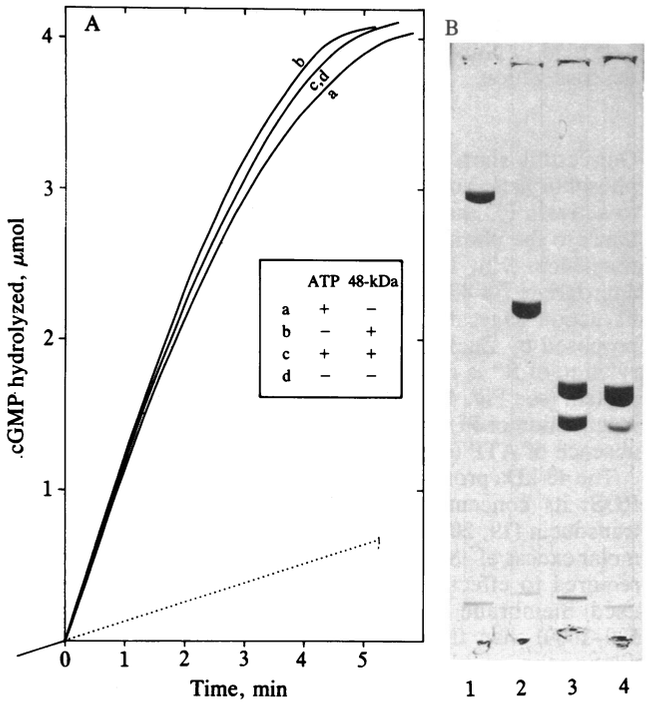

FIG. 4. (*A*) PDEase activation with urea-treated control disks: no significant effect of ATP and 48-kDa protein in the absence of rhodopsin kinase. Samples contained 5 nmol of rhodopsin, 0.5 nmol of hexylamine agarose-purified (6) transducin, 0.03 nmol of FPLC-purified (25) PDEase, 100 μM GTP, and 2.2 mM cGMP, in a final volume of 2 ml. ATP (500 μM) and/or 48-kDa protein (4 nmol) were added to samples, as indicated in the inset, before activation by a flash that bleached 4×10^{-4} of the rhodopsin. All the protein preparations were tested (9) and found to be free of rhodopsin kinase. (*B*) $NaDodSO_4$/10% PAGE of purified proteins used in reassociated systems. Lanes: 1, semipurified PDEase (before FPLC); 2, 48-kDa protein (before FPLC); 3, transducin in GDP-binding form; 4, enriched T_α–GTP[γ-S].

in (6), and urea-treated disk membranes (13)] were used, neither ATP, nor 48-kDa protein, nor a combination of both had any significant effect on PDEase activation (Fig. 4*A*).

The presence of some residual rhodopsin kinase activity most probably did not influence the results shown in Figs. 2 and 3, because these experiments were conducted in the absence of ATP. When ATP was added to P-disk samples with or without 48-kDa protein, it caused little further effect (data not shown).

No Direct Inhibition of PDEase by 48-kDa Protein. All the results presented so far are consistent with the proposed model that 48-kDa protein binds to phosphorylated R* and thereby blocks its capacity to further activate transducin and PDEase. In parallel with this two-step deactivation of R*, the active form of transducin (T_α–GTP) also becomes deactivated because of hydrolysis of the bound GTP by the intrinsic GTPase activity of transducin (2). Such removal of the active intermediate species (R*, T_α–GTP) from the cascade could be sufficient for the observed deactivation of PDEase, provided the rates of the deactivating reactions are sufficiently fast. However, the additional possibility exists that the PDEase might be directly inhibited by some ATP-dependent mechanism. To find out whether 48-kDa protein might be involved in such a mechanism, we conducted the experiments illustrated in Fig. 5.

PDEase was activated by adding long-term activated transducin (T_α–GTP[γ-S]), thus bypassing the normal activation pathway through R*. PDEase was associated to either lecithin vesicles devoid of rhodopsin (Fig. 5*A*), or to P-disks that had been kept in the dark (Fig. 5*B*). In both conditions, addition of T_α–GTP[γ-S] activated the PDEase, independently of the presence of ATP and/or 48-kDa protein. Moreover, bleaching a large fraction of rhodopsin in the P-disks during the experiment (see vertical arrow in Fig. 5*B*) had no influence on PDEase activity. This shows that 48-kDa protein, with or without ATP, has no direct influence on the activated PDEase, even in combination with phosphorylated R*.

Activating Capacity of Phosphorylated R*. The data of Figs. 2 and 3 raise the question: which component of the P-disks is predominantly responsible for the observed PDEase activation, the phosphorylated rhodopsin (a mixture of molecules of various phosphorylation levels, comprising 98% of the total rhodopsin) or the unphosphorylated rhodopsin (2% of total rhodopsin in these preparations)? Does phosphorylated R* have any activating capacity at all? Fig. 3 suggests it does, at least for low and medium light intensities, since the activity observed with P-disks (middle curve) at a given bleaching level is greater than the corresponding activity obtained with control disks at 1/50th that bleaching level, corresponding to a 2% contribution of unphosphorylated R*.

Fig. 6 shows more directly that phosphorylated R*, in the absence of any unphosphorylated R*, can indeed activate PDEase. The unphosphorylated portion was removed by anion-exchange chromatography of detergent-solubilized P-disks; the purified P-rhodopsin fraction, containing predominantly six phosphates per rhodopsin, was recombined with lecithin. After addition of transducin and PDEase, this preparation exhibited light activation of PDEase that was effectively suppressed by

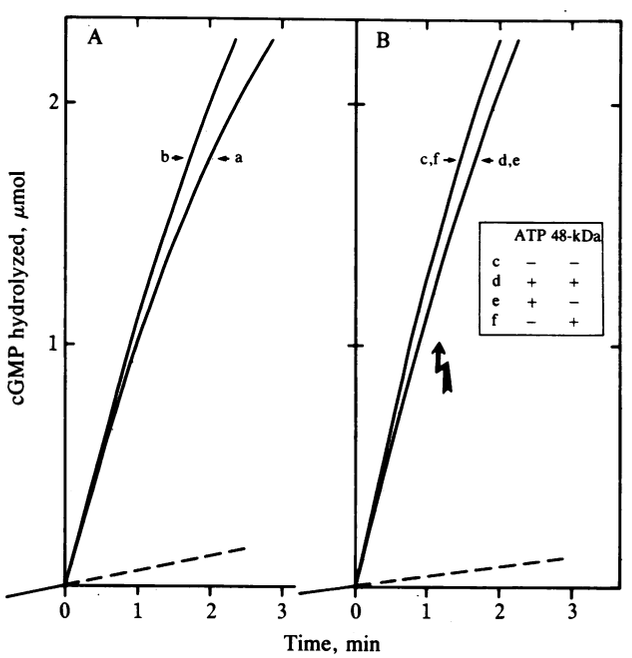

FIG. 5. PDEase activation by addition of previously activated transducin (T_α–GTP[γ-S]) in two different systems. (*A*) Purified PDEase (0.045 nmol) was added to a sonicated suspension of crude soybean lecithin (Sigma type II-S; 500 μg per sample). Fifty micrograms of 48-kDa protein and 500 μM ATP were added to sample b but not to sample a. The reaction was started by the addition of 0.07 nmol of T_α–GTP[γ-S] (see Fig. 4*B* for polypeptide pattern). (*B*) Purified PDEase (0.075 nmol) was added to dark-kept P-disks (5 nmol of rhodopsin). ATP (500 μM) and 48-kDa protein (100 μg) were added as indicated in the inset. The reaction was started by addition of 0.15 nmol of T_α–GTP[γ-S]. A flash bleaching 8.5% of the rhodopsin was delivered 1.2 min later (vertical arrow). Initial cGMP concentration was always 2 mM, and final volume, 1.4 ml. No significant effect of 48-kDa protein on PDEase activity was seen under any of these conditions; this excludes a direct inhibitory action of 48-kDa protein on the T_α–GTP[γ-S]-activated PDEase. Broken lines in *A* and *B* represent extrapolated basal activities.

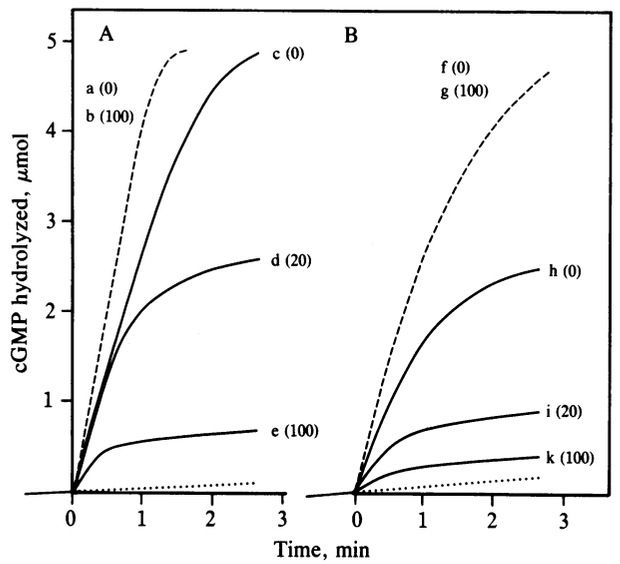

FIG. 6. PDEase activation with two different rhodopsin preparations purified in 2% octyl glucoside and recombined with lipids. Broken lines, unphosphorylated rhodopsin purified by Con A-agarose chromatography (11). Solid lines, phosphorylated rhodopsin (six phosphates per rhodopsin) purified and separated from unphosphorylated rhodopsin by ECTEOLA-cellulose chromatography (11). Each sample contained 2.3 nmol of rhodopsin (phosphorylated or not, respectively); stock solutions in 2% octyl glucoside were diluted in PDEase assay buffer containing 500 μg of sonicated crude soybean lecithin (Sigma type II-S), 0.36 nmol of transducin, 0.03 nmol of PDEase, 0.1 mM GTP, 3.5 mM cGMP, and 0.07% octyl glucoside, to a final volume of 1.4 ml. Number in parentheses indicate amounts (μg) of 48-kDa protein added to samples. The reaction was started by a flash bleaching 8% (*A*) or 0.8% (*B*) of the rhodopsin. The relatively low light-sensitivity observed with both preparations (e.g., for control rhodopsin, a bleaching level as high as 0.8% is still not saturating, compare curves f and a) is due to the reconstitution procedure.

rather small additions of 48-kDa protein (Fig. 6). Not only the initial velocity but also the turn-off time was significantly reduced by 48-kDa protein (Fig. 6).

Open Questions. The following questions are raised by the data presented.

(*i*) Does phosphorylated R*, in the absence of 48-kDa protein, really have a reduced capacity to activate membrane-associated transducin and PDEase, or is the observed reduction in activating capacity (Fig. 3) due to incomplete reassociation of the peripheral enzymes to the more negatively charged P-disks?

(*ii*) Do rhodopsin molecules phosphorylated to different extents (11) have distinct affinities for 48-kDa protein and differentially reduced PDEase-activating capacities?

(*iii*) Why does 48-kDa protein quench PDEase activation with P-disks at high bleaching levels, which produce a proportion of unphosphorylated R* (2%) supposed to be sufficient for saturating PDEase activation, but have no effect when combined with control disks at similar levels of unphosphorylated R* (compare traces b vs. e and h vs. i in Fig. 2)? It seems as if binding of 48-kDa protein to phosphorylated R* quenches PDEase activation by unphosphorylated R* molecules in some "cooperative" way. Another, more trivial explanation might be that for some reason (inverse orientation?), those rhodopsin molecules that are not available for phosphorylation are also not available for activation of PDEase; however, sonication or freeze–thaw cycles had no significant influence (data not shown).

(*iv*) In view of the numerous similarities between the photoreceptor enzyme cascade and hormone-activated adenylate cyclase systems, it seems possible that in the hormonal system, a protein related to the visual 48-kDa protein might also bind to phosphorylated receptors and thereby enhance desensitization.

CONCLUSION

Our results show that 48-kDa protein, in combination with phosphorylation of R*, quenches ("arrests") the ability of R* to activate PDEase, most probably because 48-kDa protein binds to the phosphorylated R* and thereby competes with transducin (12). The name "arrestin" (26) therefore seems appropriate for 48-kDa protein—although the mechanism of its action suggested by our experiments differs from that proposed by Zuckerman *et al.* (26). We find that phosphorylation of R* is required for the arresting action of 48-kDa protein (see Fig. 4), and we have no evidence for any direct inactivation of PDEase by 48-kDa protein in the presence or absence of ATP (see Fig. 5).

The 48-kDa protein is the most abundant soluble protein in ROS; its concentration *in vivo* may be close to that of transducin (19, 20). In our PDEase assay, a relatively large molar excess of 48-kDa protein over transducin seemed to be required to effectively quench the PDEase. However, we used membrane concentrations lower (by a factor of 500–1000) than those present *in vivo*, thereby selectively diluting the soluble 48-kDa protein, whereas the membrane-associated enzymes, transducin and PDEase, retained their high local concentrations. At *in vivo* concentrations, the situation should be much more favorable for 48-kDa protein in its competition with transducin, so that binding of 48-kDa protein to R*, after phosphorylation of R* by rhodopsin kinase, should be a very efficient mechanism to terminate the light-induced activity of R*.

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