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Transducin Inhibition of Light-Dependent Rhodopsin Phosphorylation: Evidence for $\beta\gamma$ Subunit Interaction with Rhodopsin

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SUMMARY

Rhodopsin kinase was purified from bovine retina rod outer segments as a 62–64-kDa protein that phosphorylated purified rhodopsin reconstituted into egg phosphatidylcholine/phosphatidylethanolamine liposomes. A competition binding assay in which transducin competes with rhodopsin kinase for binding sites on rhodopsin was used to assess the interaction of purified transducin subunits with rhodopsin. Preincubation of purified holotransducin with rhodopsin, in the absence of guanosine triphosphate, blocked the ability of the kinase to phosphorylate rhodopsin. Transducin-dependent inhibition of phosphorylation was relieved when guanosine 5′-(3-*O*-thio)triphosphate was present during the preincubation. Resolved α and $\beta\gamma$ transducin subunits, in the absence of guanosine triphosphate, were each capable of specifically blocking phosphorylation of rhodopsin. A maximally effective concentration of Tα or T$\beta\gamma$ (1 μM) subunits inhibited phosphorylation of rhodopsin (0.23 μM) 45–65%. A similar concentration of reconstituted transducin (Tα and T$\beta\gamma$) or native holotransducin (T$\alpha\beta\gamma$) inhibited phosphorylation greater than 98%. The results indicate that rhodopsin must have a binding site for T$\beta\gamma$ as well as a binding site for Tα, and each subunit influences the recognition of bleached rhodopsin by rhodopsin kinase.

Rhodopsin belongs to a large family of receptor proteins that function as exchange catalysts leading to the activation of specific G proteins (1). In the retina rod outer segment rhodopsin binds and activates transducin, the G protein that regulates cyclic GMP phosphodiesterase (2). Striking structural and functional homology exists between rhodopsin and β-adrenergic (3, 4) and muscarinic (5) receptors as well as between transducin and the other G proteins including G_s, G_i, and G_o (6–11). An additional property common to receptors that activate regulatory G proteins is that they are phosphorylated by specific kinases when bound by an agonist (12) or, in the case of rhodopsin after absorption of a photon (13, 14).

The interactions of the Tα and T$\beta\gamma$ subunits of transducin with rhodopsin remain poorly defined (15, 16). In fact, the data of Fung (17, 18), using [^{14}C]ethyl acetimidate-derivatized Tα or T$\beta\gamma$ and the separation of components by centrifugation, demonstrated that Tα interacted poorly with rhodopsin and a direct interaction of T$\beta\gamma$ with the bleached photoreceptor could not be demonstrated. Based on these experiments, Fung (17) postulated that T$\beta\gamma$ stimulated Gpp(NH)p binding and GTPase activity by facilitating Tα interactions with rhodopsin. We have used the light-dependent phosphorylation of rhodopsin by rhodopsin kinase, which is shown to be inhibited by the binding of transducin to rhodopsin, to study the interaction of transducin subunits with the receptor. In contrast to the assay-system used by Fung (17, 18), this approach allows use of nonderivatized native transducin subunits and does not require centrifugation or any other technique for separation of free from bound transducin, which could fail to detect an interaction if very rapid dissociation rates exist. The findings indicate that T$\beta\gamma$, as well as Tα, has a specific binding site on the rhodopsin molecule and that interaction of rhodopsin with either subunit diminishes receptor phosphorylation.

Methods

Preparative methods. Rod outer segment membranes were isolated in the light as previously described (19) except that the step gradients used were 25% and 30% (w/w) sucrose as suggested by Sitaramayya (20) and leupeptin, aprotinin, and phenylmethylsulfonyl fluoride were included in the buffers. The membranes were then ex-

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ABBREVIATIONS: G proteins, guanyl nucleotide binding regulatory proteins; G_s and G_i, stimulatory and inhibitory G proteins of adenylate cyclase; G_o, G protein of brain; T, transducin G protein of rod outer segments; Tα and T$\beta\gamma$, subunits of transducin; Gpp(NH)p, guanyl-5′-yl-imidophosphate; GTPγS, guanosine-5′-(3-*O*-thio)-triphosphate; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

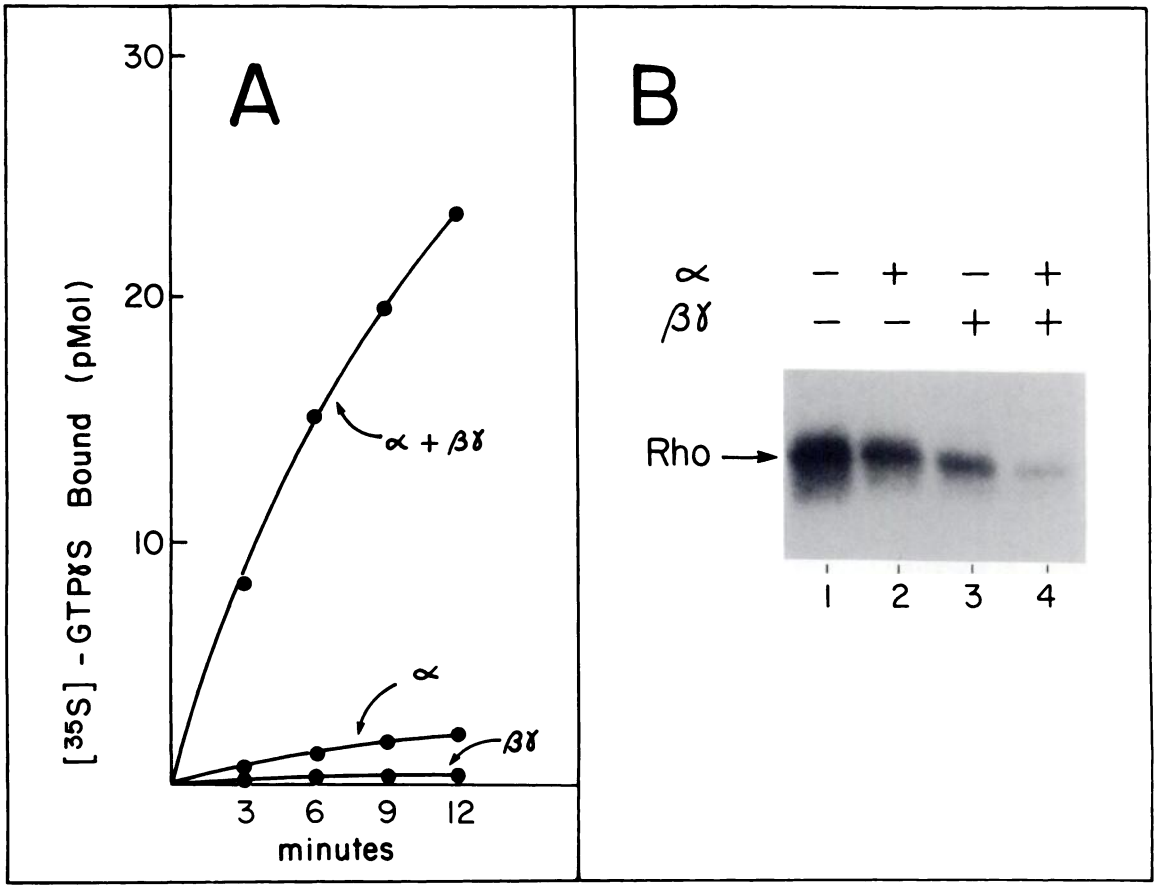

Fig. 1. Ability of Tβγ to stimulate the rate of rhodopsin-dependent activation of Tα and to block phosphorylation of bleached rhodopsin. Purified Tβγ and Tα subunit preparations eluted from blue Sepharose were used in [^{35}S]GTPγS binding assays (A) or in assays testing their ability to block phosphorylation of purified reconstituted rhodopsin (*Rho*) (B) by purified rhodopsin kinase. A, [^{35}S]GTPγS binding of the Tα and Tβγ preparations (0.3 μM each) was measured as a function of time as shown in the presence of light and 0.01 μM purified rhodopsin. The [^{35}S]GTPγS concentration was 0.5 μM (34,000 cpm/pmol). Nonspecific binding was measured as [^{35}S]GTPγS cpm bound to BA85 nitrocellulose filters in the presence of phospholipid vesicles plus or minus reconstituted purified rhodopsin and was not significantly different from that shown for βγ in the presence of reconstituted rhodopsin and was not subtracted from the data shown. The rate of binding of [^{35}S]GTPγS to Tα (plus or minus βγ) in the absence of rhodopsin (not shown) was very low but detectable and is reported in the text. B, Autoradiograph of [^{32}P]P_i incorporation into rhodopsin. Purified rhodopsin (0.23 μM) was preincubated for 30 min on ice in the dark alone (*lane 1*) or in the presence of 0.3 μM Tα (*lane 2*) or 0.3 μM Tβγ (*lane 3*) or 0.3 μM of each transducin subunit (*lane 4*). Purified rhodopsin kinase (15 μl) and [γ-^{32}P]ATP (10 μM, 12,200 cpm/pmol) was then added to all samples, which were incubated an additional 5 min at 23° in the light before addition of SDS sample buffer and electrophoresis on 10% acrylamide gels. The gels were Coomassie stained, destained, and dried and autoradiographs were prepared. The autoradiograph (B) shows only the region of the gel containing the Coomassie-stained, ^{32}P-labeled, 34-kDa rhodopsin monomer inasmuch as the rhodopsin dimer was essentially nondetectable either by Coomassie staining or [^{32}P]P_i content (for example see Fig. 4, *3-min lane*). Per cent inhibition of rhodopsin phosphorylation (see text) was calculated based on Cerenkov counting of the excised bands from three separate experiments. Other final concentrations in both 100-μl assays (A and B) were 10 mM Tris·HCl, pH 7.4, 0.04 mM EDTA, 0.04 mM EGTA, 2.5 mM $MgCl_2$, 274 mM NaCl, and 2.1 mM DTT. All assays were initiated within 1 hr of isolation of the purified transducin subunits and rhodopsin kinase. At this time essentially all of the α subunit was fully functional, based upon its ability to bind 1 mol of [^{35}S]GTPγS per mol when the binding reaction had reached steady state (not shown). The ability of the Tβγ subunit preparation to stimulate rhodopsin-dependent [^{35}S]GTPγS binding to Tα remains stable for days at 0° (not shown). Note also that in the experiments reported in B no guanine nucleotide was added.

tracted for 60 min on ice using 200 mM NaHEPES, 20 mM EDTA, 2 mM DTT, pH 8.0, followed by centrifugation (100,000 × *g*, 15 min) (21). The supernatant is referred to as rhodopsin kinase extract. The membranes were washed and transducin was then extracted and purified and the subunits were resolved as previously described (19), except that the blue-Sepharose buffer for pre-equilibration and washing buffer contained 210 mM NaCl in place of 100 mM NaCl, and Tα was eluted with the same buffer but containing 635 mM NaCl.

The rhodopsin kinase extract was dialyzed against 10 mM Tris·HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM DTT (buffer A) and loaded onto a DEAE-cellulose column equilibrated in buffer A. The column was washed with 20 column volumes and rhodopsin kinase was eluted using a 35–135 mM NaCl gradient in buffer A. Rhodopsin kinase-containing fractions, assayed by phosphorylation of bleached rhodopsin (13, 14, 21), were then loaded directly onto a blue-Sepharose column equilibrated in 75 mM NaCl and buffer A. Rhodopsin kinase was eluted using a 100–500 mM NaCl linear gradient in buffer A and elutes at approximately 310 mM NaCl as a 62–64-kDa band when analyzed on SDS-acrylamide gels.

Rhodopsin was extracted, using 1.5% octyl glucoside, from membranes from which rhodopsin kinase and transducin had been extracted as described above, except that the entire procedure was done under dim red light. Rhodopsin was purified by chromatography on Concanavalin A-Sepharose (22) and reconstituted into egg phosphatidylcholine/phosphatidylethanolamine vesicles (17) by dialysis against 10 mM Tris·HCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT after addition of 5 mM EDTA to the rhodopsin/lipid mixture.

Other methods. The protein concentration of purified preparations of Tα, Tβγ, rhodopsin, and Tαβγ were estimated by Bradford protein assay (23), relative to bovine serum albumin standards, which yielded

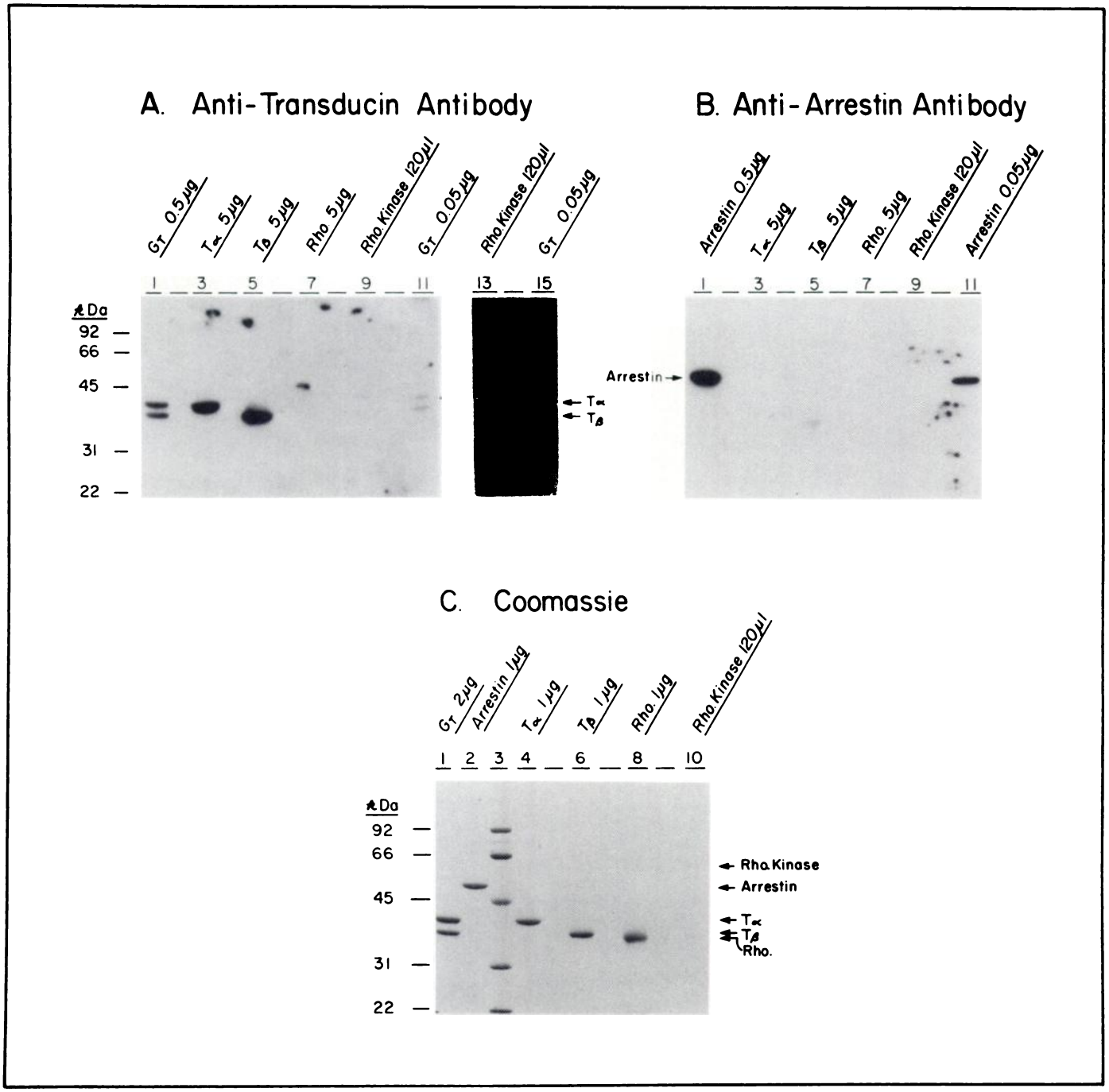

Fig. 2. Characterization of transducin subunit, rhodopsin, and rhodopsin kinase preparations by Western blot analysis. The purity of the four protein preparations with respect to contaminating transducin subunits or arrestin was determined by ^{125}I-protein A Western blot analysis using a polyclonal (rabbit) anti-holotransducin antibody (A) or a monoclonal (mouse) anti-arrestin antibody (B) to probe 5-µg quantities of Tα (*lanes 3*), Tβγ (*lanes 5*), or rhodopsin (Rho; *lanes 7*) and 120 µl of rhodopsin kinase (*Rho. Kinase;* approximately 0.12 µg of kinase) (*lanes 9* and *13*) after electrophoresis on 10% acrylamide/0.25% bis-acrylamide gels and transfer to nitrocellulose. The autoradiographic response to smaller amounts of the positive controls holotransducin (G_T, 0.5 µg, *lane 1*; 0.05 µg, *lanes 11* and *13*; A) and arrestin (0.5 µg, *lane 1*; 0.05 µg, *lane 11*; B) are shown. Low molecular weight standards were run in all even-numbered lanes between samples and a nonspecific signal from the 66-kDa standard (1 µg) can be seen in A. *Lanes 13–15* in A are equivalent to *lanes 9–11* and represent a duplicate blot of the same kinase preparation. These lanes are shown due to the artifact visible in *lane 9* just above the mobility corresponding to Tα. The anti-arrestin antibody interacted with low affinity with Tβ (5 µg of Tβ, *lane 5*) compared with arrestin itself (0.05 µg of arrestin, *lane 11*). Conditions were optimized for maximal sensitivity, which is reported in the text. For detection of Tα or Tβ, transferred proteins (20 min, 380 mA, 0.75 mm gel) were incubated for 1 hr (23°) with blocking buffer (10 mM Tris·HCl, 7.4, 150 mM NaCl, 10% γ-globulin-free horse serum) then incubated overnight with primary antibody (1:200 dilution) in blocking buffer. After washing (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Tween 20) the nitrocellulose was incubated for 1 hr with ^{125}I-protein A (2 nM, 10^6 cpm/pmol) in blocking buffer, then washed again, air-dried, and autoradiographed 4–5 hr. The protocol for detection of arrestin was similar except that transfer time was 45 min and the primary antibody (1:400) incubation was followed, after washing, by incubation with secondary rabbit-anti-mouse IgG antibody (1:1000) in blocking buffer then washed before incubation with ^{125}I-protein A. For reference, C shows a representative Coomassie-stained 10% gel of 1 µg/band amounts of all the protein preparations used except that 120 µl of a rhodopsin kinase preparation is shown (also see Fig. 4, *lane III*). *Lane 3* (C) was loaded with low molecular weight standards (1 µg each; see Methods). Note that no more than 15 µl of kinase preparation per 100 µl assay was used in the experiments such as those described in Figs. 1 and 5.

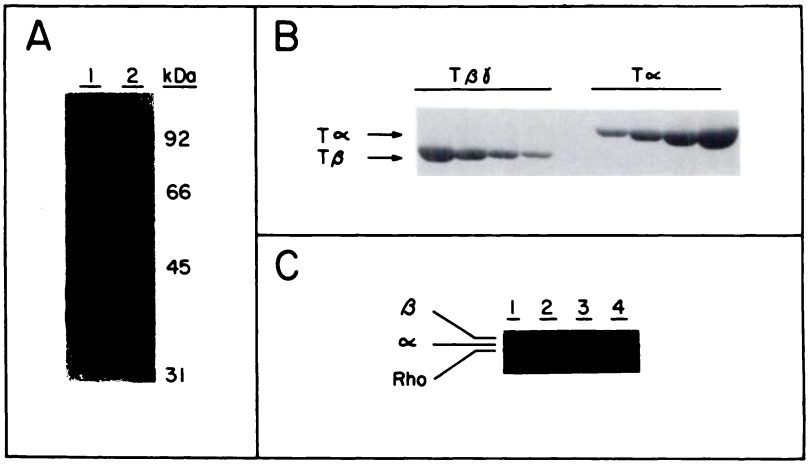

Fig. 3. Characterization of transducin subunit and purified reconstituted rhodopsin preparations by Coomassie staining of SDS-acrylamide gels. Coomassie-stained 10% acrylamide/0.25% bis-acrylamide gels (A and B) loaded with up to 30 μg of hexyl-agarose-purified holotransducin or 15 μg of the transducin subunit preparations, which were derived by chromatography of the purified holotransducin on blue Sepharose, was one method used to assess the purity of these preparations. A shows a representative holotransducin preparation (26 μg) on a gel from which the dye front was run off to improve resolution of large amounts of Tα and Tβ. B shows the 34–42-kDa region of a similar gel loaded with 1.2, 2.4, 4.8, or 9.6 μg of Tβγ and 1.5, 3.0, 6.0, or 12 μg of Tα. The purity of the rhodopsin (*Rho*) preparation, in particular potential contamination with Tα, was monitored by loading up to 7 μg of rhodopsin in the same gel system (not shown). Larger amounts of rhodopsin could not be resolved from transducin. Potential contamination of the rhodopsin preparation with Tβγ was similarly assessed but on 15% acrylamide/0.08% bis-acrylamide gels (C) as described by Baehr *et al.* (32), to improve resolution of Tβ (*lane 2*, 1.5 μg) from purified rhodopsin (*lane 1*, 1 μg). For reference, holotransducin (*lane 3*) and purified Tα (*lane 4*) are shown, indicating the reversal of mobilities of Tβ and Tα in this gel system (compare C and B). Results and detection limits are reported in the text.

values essentially the same as those derived from Coomassie staining of electrophoresed bands, relative to electrophoresed albumin standards quantified by densitometry. Molar concentrations were estimated using molecular weight values of 39,000, 42,000, 39,000, and 81,000 for Tα, Tβγ, rhodopsin, and Tαβγ, respectively. For the Tα preparations used in these studies, concentrations estimated as described above were within 20% of values derived from steady state binding levels of [^{35}S]GTPγS, assuming 1:1 stoichiometry. The concentration of purified rhodopsin kinase is reported as microliter of kinase preparation per stated assay volume. All kinase preparations used in these studies contained 0.09–0.12 μg of Coomassie-stained 62 kDa band and 0.2–0.3 μg of total protein per 100 μl.

Transducin [^{35}S]GTPγS binding assays have been previously described (21, 24).

The low molecular weight markers used on SDS-acrylamide gels were as follows: phosphorylase *b*, 92 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 22 kDa; and lysozyme, 14 kDa.

Results

Activation of transducin by bleached rhodopsin involves the following three steps: binding of transducin to bleached rhodopsin, release of GDP from Tα and the binding of GTP, and dissociation of Tα·GTP from Tβγ (2, 25, 26). Rhodopsin kinase has also been shown to bind to bleached rhodopsin and phosphorylate serine and threonine residues near the carboxyl-terminus of opsin (27–31). Thus, after bleaching, transducin and rhodopsin kinase may compete for interaction with rhodopsin. Based on these observations, we have utilized the specific interaction and phosphorylation of bleached rhodopsin with rhodopsin kinase as an assay to monitor Tα and Tβγ interactions with the cytoplasmic domain of rhodopsin.

Fig. 1A shows that Tβγ must be present for efficient rhodopsin-catalyzed activation of GTPγS binding to Tα. In the presence of bleached rhodopsin, addition of Tβγ enhances the *rate* of [^{35}S]GTPγS binding to Tα, an indication of the functionality of both the Tβγ and Tα preparations. In the presence of bleached rhodopsin and Tβγ alone (Fig. 1A, βγ) the level of [^{35}S]GTPγS binding shown was not distinguishable from nonspecific binding observed in the presence of vesicle-reconstituted rhodopsin alone or vesicles alone. In the absence of bleached rhodopsin and Tβγ, the Tα preparation used in this experiment specifically bound [^{35}S]GTPγS at a slow but detectable rate of 0.03 pmol min^{-1} (not shown). Addition of bleached rhodopsin (0.01 μM) increased the rate of [^{35}S]GTPγS binding to 0.15 pmol min^{-1}. This observation that a low concentration of purified rhodopsin can increase the rate of binding of [^{35}S]GTPγS to purified Tα in the absence of detectable Tβγ (see below) is consistent with the notion that Tα can bind to rhodopsin in the absence of Tβγ but that catalysis of guanine nucleotide exchange is inefficient (17). The results shown in Fig. 1A and those reported above were confirmed in at least six separate experiments.

Using the same preparations of Tα and Tβγ as that used to measure [^{35}S]GTPγS binding, Fig. 1B shows that Tα or Tβγ alone were capable of partially inhibiting rhodopsin phosphorylation by rhodopsin kinase. Mixing of Tα and Tβγ resulted in a near complete inhibition of rhodopsin phosphorylation. In three independent experiments, the percent inhibition due to 0.3 μM Tα, Tβγ, or Tα and Tβγ was 38 ± 15, 50 ± 10 and 76 ± 5, respectively (mean ± standard error, three experiments).

This finding indicated that both Tα and Tβγ can independently interact with bleached rhodopsin and alter its ability to serve as a substrate for rhodopsin kinase. This result and interpretation requires that the transducin subunits have been resolved from one another, as well as from rhodopsin and rhodopsin kinase, and that Tα and Tβγ did not inhibit phosphorylation of rhodopsin by direct interaction with rhodopsin kinase.

Western blot analysis (Fig. 2) provided the most sensitive confirmation that the four kinds of purified protein preparations used were free of contaminating transducin subunits. Five micrograms of purified Tα, Tβγ, or rhodopsin and 120 μl of the rhodopsin kinase preparations used in these studies were probed with a polyclonal antibody that recognized both Tα and Tβ. The sensitivity of this antibody was similar for Tα and Tβ and could recognize as little as 0.01 μg of Tα or Tβ. Fig. 2A, *lanes 11* and *13* show detection of 0.025 μg of Tα or Tβ in two separate blots. Contaminating transducin subunits were not

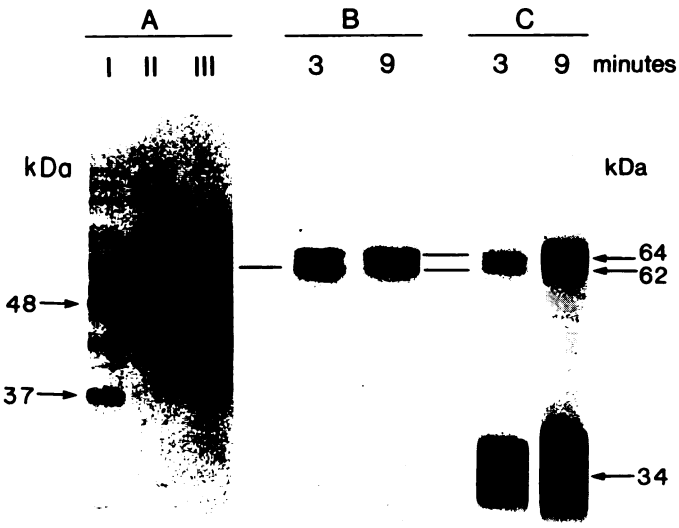

Fig. 4. Purified rhodopsin kinase phosphorylates purified rhodopsin in reconstituted vesicles and is detected as an autophosphorylated 62–64-kDa doublet. Rhodopsin kinase was purified by sequential chromatography on DEAE-cellulose and blue-Sepharose as described in the Methods. A shows the Coomassie blue staining profile of the rhodopsin kinase extract resolved on SDS-PAGE (*I*), DEAE-cellulose-pooled peak fractions (*II*), and the pooled blue-Sepharose eluates (*III*). One hundred and twenty microliters of each preparation were loaded onto the gels corresponding to 4.6, 1.5, and 0.28 μg of total protein, respectively. The band at 48 kDa in *lane I* was identified as arrestin by Western blot (not shown). The band at 37 kDa in *lane I* has not reacted with transducin antibodies and remains undefined. B shows an autoradiograph of the blue Sepharose-purified rhodopsin kinase preparation (80 μl) after incubation with 50 μM [γ-^{32}P]ATP (5000 dpm/pmol) for 3 or 9 min. Rhodopsin kinase corresponds to a 62–64-kDa protein and undergoes a time-dependent autophosphorylation that influences its migration on SDS-PAGE. Ten percent acrylamide/0.25% bis-acrylamide gels were used and the dye front was allowed to run off in order to detect the autophosphorylated kinase as a doublet. C has the same conditions as in B except for the addition of 1 μM bleached rhodopsin, which is phosphorylated in a time-dependent manner by rhodopsin kinase. In the experiment shown, 21,700 dpm above background were incorporated into the rhodopsin monomer (34 kDa) after 3-min incubation. Note that only a very small fraction of the [^{32}P]P_i incorporated into rhodopsin is detected as rhodopsin dimer, which migrates as a rather broad band together with the autophosphorylated kinase doublet. After addition of SDS sample buffer to terminate phosphorylation reactions, the samples were neither heated nor frozen, to minimize artifactual formation of phosphorylated rhodopsin dimer, which would obscure detection of the authophosphorylated kinase doublet on autoradiographs. The assay (100 μl) also contained 11 mM Tris·HCl, pH 7.4, 0.95 mM EDTA, 0.85 mM EGTA, 3.2 mM $MgCl_2$, 263 mM $NaCl_2$, and 2.2 mM DTT. Unbleached rhodopsin is not phosphorylated by rhodopsin kinase under these conditions (not shown).

detected in any of the four protein preparations (Fig. 2A). Thus, the Tα, T$\beta\gamma$, and rhodopsin preparations were greater than 99.8% pure with respect to contaminating transducin subunit. These data also indicate that the kinase preparation contains less than 0.01 μg of Tα or Tβ per 120 μl of kinase preparation. Because this is equivalent to about 0.002 μM maximal contaminating Tα or T$\beta\gamma$ in the kinase preparation and the kinase preparation was used at a dilution of at least 6.6-fold (see Figs. 1 and 5), the maximal Tα and Tβ contamination in the final assays would be less than 0.3 nM. This concentration is at least 2 orders of magnitude below the minimal concentrations of either Tα or T$\beta\gamma$ that were effective in inhibiting rhodopsin phosphorylation in the presence of 0.23 μM rhodopsin (see Fig. 5).

Fig. 2 also demonstrates (B) that the Tα, T$\beta\gamma$, rhodopsin, and rhodopsin kinase preparations were not contaminated by traces of arrestin, a retinal protein that also interacts with rhodopsin and could potentially form a ternary complex with transducin subunits because it has limited homology with Tα. The sensitivity of the monoclonal anti-arrestin antibody used was about 0.005 μg of arrestin. Fig. 2B, *lane 11* shows a response to 0.05 μg of arrestin. As for the transducin blots, 5-μg quantities of Tα, T$\beta\gamma$, and rhodopsin and 120 μl of the kinase preparation were probed with this antibody (Fig. 2B). Therefore, because each of the four preparations is free of detectable arrestin, it is clear, for example, that a T$\beta\gamma$ interaction with rhodopsin in the present studies is not mediated by a ternary complex formation involving arrestin.

The purity of the protein preparations used in these studies was also monitored by Coomassie staining of samples electrophoresed in the SDS-acrylamide gel systems described in the legend to Fig. 3. Neither arrestin nor contaminating transducin subunits were observed when 15 μg of Tα, 15 μg of T$\beta\gamma$, 7 μg of rhodopsin, or 120 μl of kinase was examined. The detection limit was 0.07 μg of Tα, Tβ, or arrestin. During the purification of T$\beta\gamma$, Tγ remained tightly associated with Tβ inasmuch as the γ subunit abundance relative to the abundance of the β subunit was similar for the T$\beta\gamma$ preparation, compared with the holotransducin preparation. This was observable by Coomassie staining of 10–15-μg amounts of T$\beta\gamma$ and holotransducin preparations electrophoresed on 10% acrylamide gels from which the dye front was not run off (not shown).

Recently described preparations (membrane extracts) of rhodopsin kinase have not been amenable to additional purification (20, 30, 31), perhaps due to low initial concentrations of the kinase, which is not the case using our extraction procedure (21). The results of chromatography of the rhodopsin kinase extract on DEAE followed by blue Sepharose are shown in Fig. 4. These chromatographic steps were particularly important to remove a major band of arrestin (48 kDa) present in the original extract (Fig. 4, *lane I*) as well as a small but significant amount of transducin from the original kinase extract. The abundant 37-kDa protein of unknown function that was present in our original extract (Fig. 4, *lane I*), as well as those of others (20, 30, 31), was also removed by this chromatography procedure. Purified rhodopsin kinase appeared as a 62-kDa band by Coomassie staining after electrophoresis of 120-μl portions of the final preparation on 10% acrylamide gels (Fig. 4, *lane III*), similar to the molecular mass of the enzyme reported by Sitaramayya (20). The enzyme undergoes a time-dependent autophosphorylation in the presence or absence or rhodopsin (Fig. 4), which appears to influence its mobility on acrylamide gels and is detectable, as indicated by the autoradiographs shown, as a 62–64-kDa doublet if the samples are prepared and electrophoresed as described in the legend to Fig. 4. In the purified kinase preparation, which was about 50% pure (Fig. 4 and legend), the only ATP-binding proteins detectable by ATP analogue affinity cross-linking were the rhodopsin kinase band(s) (not shown).[2] Autophosphorylation of rhodopsin kinase occurs only on serine residues and does not influence the affinity of the enzyme for its substrate rhodopsin.[2]

The results presented in Fig. 5 and 6 characterize properties of the transducin inhibition of rhodopsin phosphorylation and provide several types of indirect evidence that the transducin-

[2] D. J. Kelleher and G. L. Johnson, manuscript in preparation.

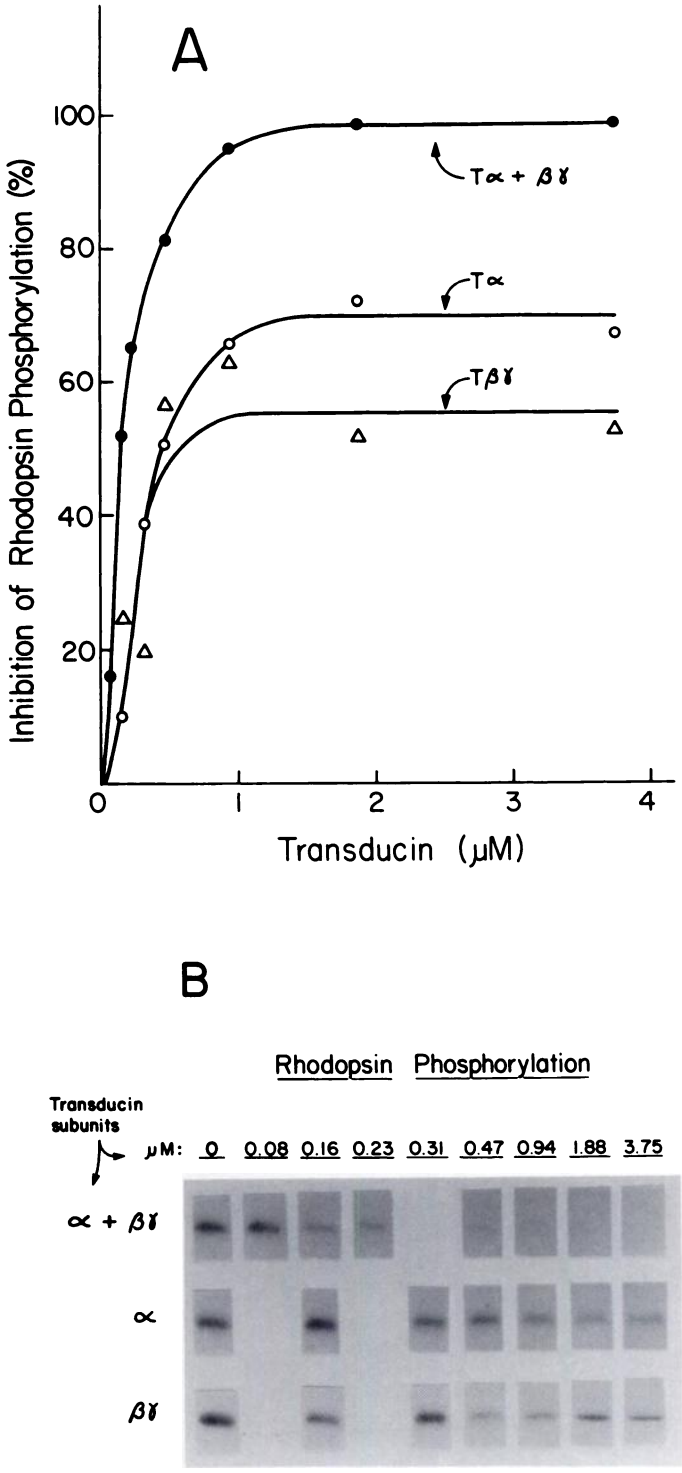

Fig. 5. Concentration dependent ability of Tβγ and Tα to block light-dependent phosphorylation of rhodopsin. The protocol was as described in detail in the legend to Fig. 1B, except that the concentration of transducin subunits was varied between 0 and 3.75 μM. The concentrations of purified Tα, Tβγ, and rhodopsin were based upon Bradford protein assay (bovine albumin standard) and molecular weights of 39,000, 42,000, and 39,000, respectively (see Methods). The data presented (A) were calculated from Cerenkov cpm (50% counting efficiency) of [^{32}P]P$_i$ incorporated into the Coomassie-stained rhodopsin monomer bands excised from the dried 10% acrylamide gels. A backround value of less than 100 cpm for each of three gels was determined by counting backround pieces of the gels of size similar to the rhodopsin bands, and these values were subtracted. The data points shown represent single determinations at each transducin concentration and are expressed as per cent inhibition of control phosphorylation. Radioactivity (dpm) incorporated into rhodopsin in the absence of transducin (5110 ± 550, mean ± standard deviation, three determinations) served as the control. The data points at the two highest Tα concentrations were averaged, as were the values of the four highest Tβγ data points, in order to estimate a plateau level and smooth curves for Tα and Tβ were drawn to these plateau levels. The results shown are representative of two separate experiments and autoradiographs of the rhodopsin bands are shown in B.

dependent inhibition of rhodopsin phosphorylation is mediated by the interaction of transducin with rhodopsin and not the kinase.

Fig. 5 shows the concentration dependence for Tα and Tβγ to alter the ability of rhodopsin to serve as a substrate for rhodopsin kinase. The effect of Tα and Tβγ was a saturable process and neither subunit alone was capable of inhibiting rhodopsin phosphorylation to the extent observed with the combination of Tα and Tβγ. Maximal inhibition occurred at approximately 1 μM Tα or Tβγ, which was approximately a 4-fold excess over the rhodopsin concentration (0.23 μM). The kinase concentration was about 0.0024 μM. Half-maximal inhibition by reconstituted holotransducin (Tα and Tβγ) occurred at 0.15 μM, compared with 0.3 μM for Tα or Tβγ alone. The saturation curve for inhibition of rhodopsin phosphorylation was similar for native holotransducin (not shown), compared with that shown for the reconstituted protein. These values do not provide an estimate of the binding affinity of the transducin subunits with rhodopsin but do indicate that inhibition of phosphorylation of rhodopsin requires approximately

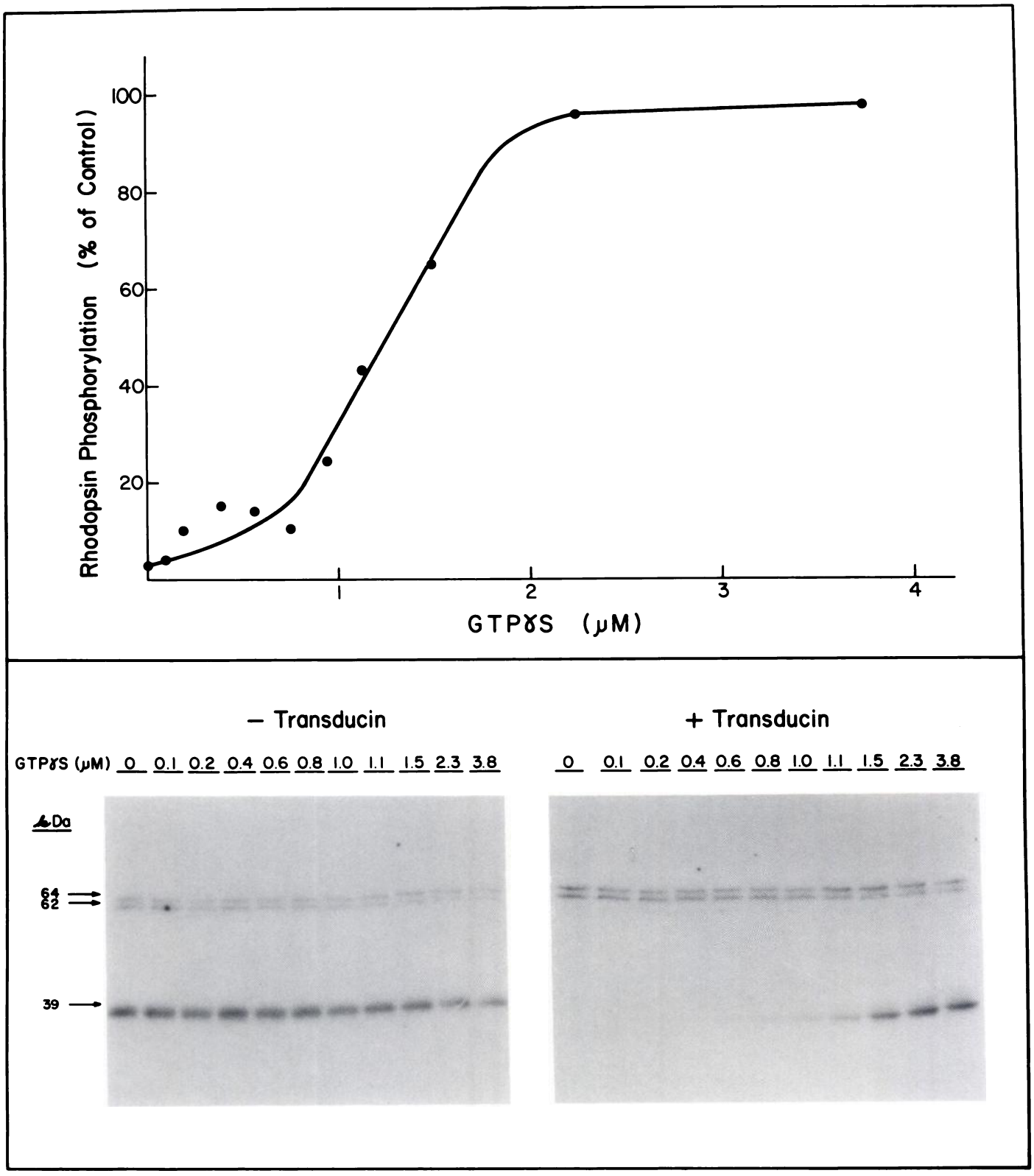

Fig. 6. Reversal of transducin inhibition of rhopsin phosphorylation by GTPγS. Purified reconstituted rhodopsin (0.45 μM) was preincubated for 15 min at 23° in the light in the presence or absence of purified holotransducin (1.2 μM) and the presence or absence of increasing concentrations of GTPγS (0.1–3.8 μM). Forty microliters of purified rhodopsin kinase and 10 μl of [γ-^{32}P]ATP (10 μM final concentration) was then added. The volume was then 200 μl. After 5-min additional incubation, SDS sample buffer was added to stop the reaction. Incorporation of [^{32}P]P_i into the 34-kDa Coomassie-stained rhodopsin band was determined by autoradiography of 9% acrylamide/0.23% bis-acrylamide gels (*lower*) and Cerenkov counting of the excised bands. Each condition was run in duplicate (only two of four autoradiographs are shown) and the appropriate samples, incubated in the absence of transducin, served as the control for the sample incubated in the presence of transducin. Extent of phosphorylation in the presence and absence of transducin was determined and this ratio, expressed as percentage of control, was plotted as a function of GTPγS concentration (*upper*). Note that even the lowest concentrations of GTPγS present during the preincubation (0.1 μM) is above the K_d for GTPγS binding to rhodopsin-preactivated transducin (0.05 μM GTPγS; Ref. 24) and that the concentration of GTPγS required for 50% reversal of transducin inhibition of rhodopsin phosphorylation (*upper*) is not a measure of GTPγS binding affinity. Rather, this value is in the range of 1–2 μM GTPγS and corresponds approximately to the concentration of transducin added.

stoichiometric amounts of transducin subunit compared with the amount of rhodopsin present. This is an indication that the inhibition of rhodopsin phosphorylation depends on the interaction of transducin with rhodopsin rather than on interaction of transducin with the kinase. Estimates of the dissociation constant for the interaction of holotransducin·GDP and rhodopsin of 0.2 μM (33) and 0.05 μM (34) have been previously reported. It should be noted that the inhibition of rhodopsin phosphorylation is highly specific to transducin and its subunits. Rabbit serum at concentrations up to 2.3 mg of protein/ml did not influence rhodopsin phosphorylation. This is in comparison with 1 μM Tβγ, which represents approximately 0.04 mg of protein/ml. Also, guanine nucleotides at concentrations up to 10 μM, in the absence of transducin, had no influence on rhodopsin kinase activity.

Incubation of holotransducin (1.2 μM) with bleached rhodopsin (0.45 μM) results in a stable complex between the two proteins (34). Under these conditions, rhodopsin phosphorylation by rhodopsin kinase was inhibited more than 98% (Fig. 6). With increasing concentrations of GTPγS, the ability of transducin to block rhodopsin phosphorylation was progressively lost. Reversal of transducin's inhibition of rhodopsin phosphorylation closely paralleled the binding of GTPγS to transducin, detected by filter binding assays (not shown), which has been shown to induce dissociation of transducin from bleached rhodopsin (2). Thus, GTPγS binding to transducin, which was dependent upon and catalyzed by its interaction with bleached rhodopsin, dissociated the rhodopsin·transducin complex and the receptor became available as a substrate for rhodopsin kinase. This provides additional evidence that the ability of transducin to inhibit phosphorylation depends on the ability of transducin to interact with rhodopsin rather than an interaction of transducin with the kinase. Fig. 5B also shows that autophosphorylation of rhodopsin kinase (the 62–64-kDa doublet, see Fig. 4)[2] was not inhibited by the presence of transducin. This also indicates that the inhibition of rhodopsin phosphorylation by transducin is due to the interaction of transducin with bleached rhodopsin and not an effect on rhodopsin kinase.

Discussion

We have demonstrated that purified Tβγ or Tα can independently block phosphorylation of rhodopsin. The effect of Tα and Tβγ was to alter rhodopsin as a substrate by their direct interaction with the rhodopsin molecule. Three lines of evidence support this interpretation. First, the ability of transducin or its purified subunits to inhibit rhodopsin phosphorylation is a saturable, concentration-dependent process, reaching a maximum value when the transducin concentration is 1 μM and the rhodopsin concentration is 0.23 μM. Second, the ability of transducin to inhibit rhodopsin phosphorylation is relieved as a result of the rhodopsin-catalyzed binding of GTPγS to transducin. Third, transducin can completely inhibit rhodopsin phosphorylation without affecting autophosphorylation of the kinase.

The implications of a Tβγ binding site on rhodopsin are 2-fold. First, the mechanism of receptor activation of GTP-binding regulatory proteins must accommodate a role of Tβγ interactions with rhodopsin. Second, the dissociation model of Gilman (1) in which βγ subunits have been shown to dissociate from the α·GTP subunit complex *in vitro*, predicts that if a similar dissociation event occurs in the cell, then free βγ subunits would be released during photoexcitation in the retina or hormonal stimulation of the adenylate cyclase system. Free βγ subunits would be predicted to interact with receptors and alter the ability of specific kinases to phosphorylate activated receptors. Free Tβγ subunits thus have the ability of altering the rhodopsin phosphorylation at the serine-threonine-rich carboxy-terminus, which has been implicated in adaptation in the rhodopsin-transducin cascade in the rod outer segment (27, 30).

It should also be noted that GTPγS completely reversed the transducin inhibition of rhodopsin phosphorylation. The degree of GTPγS reversal of the phosphorylation inhibition was greater than predicted if only the Tα component of the inhibition was lost by the formation of Tα·GTPγS. It was predicted that Tβγ should still influence the phosphorylation of rhodopsin by rhodopsin kinase. In fact, when Tβγ and Tα·GTPγS were added to bleached rhodopsin, in contrast to being generated during the assay as in the experiment shown in Fig. 6, the Tβγ component of the inhibition of rhodopsin phosphorylation was observed (data not shown). It appears, therefore, that preincubation of bleached rhodopsin with holotransducin (Tαβγ) and subsequent binding of GTPγS prevents the inhibition of rhodopsin phosphorylation by Tβγ. One possibility is that binding of holotransducin to bleached rhodopsin induces or stabilizes a conformation of the bleached rhodopsin that no longer recognizes either free Tα·GTPγS or free Tβγ subunits but readily recognizes rhodopsin kinase. If this were the case, then Tβγ would only effectively inhibit phosphorylation of bleached rhodopsin that had not bound and released activated transducin.

Our finding that Tβγ directly interacts with the rhodopsin molecule was substantiated by Halpern *et al.* (35), who raised a rabbit antiserum against Tβγ that contained antibodies to rhodopsin. The antibodies were not in preimmune serum and were postulated to represent antiidiotypic antibodies raised against idiotypic anti-Tβγ antibodies. This result also suggested that Tβγ has a binding site for rhodopsin. Thus, Tβγ must contribute to the catalytic activation by receptor of Tα, by interaction with both receptor and Tα. Defining these binding sites will be essential in elucidating the role of Tβγ in receptor-G protein signalling pathways and their regulation by receptor phosphorylation.

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