A C-terminal peptide of bovine rhodopsin binds to the transducin α -subunit and facilitates its activation

William J. PHILLIPS* and Richard A. CERIONE

Department of Pharmacology, Schurman Hall, Cornell University, Ithaca, NY 14853, U.S.A.

In order to investigate the possible roles of the intracellular domains of rhodopsin in the functional coupling of the photoreceptor to transducin, different peptides that correspond to parts of the known sequence of rhodopsin were synthesized. Since we have found that the binding of rhodopsin to the α subunit of transducin (α_T) increases the susceptibility of α_T to phosphorylation by protein kinase C- β_1 , we used this phosphorylation reaction as an initial screen for peptides that mimic the actions of rhodopsin. The results of this screen indicated that a peptide from the C-terminal tail of rhodopsin (amino acids 325–338; KNPLGDDEASTTVS-amide; designated as peptide 3) was capable of interacting with the α_T subunit. Evidence that peptide 3 binds to α_T at a site that overlaps the rhodopsin-binding domain was obtained from experiments showing that

peptide 3 inhibited the rhodopsin-stimulated GTPase activity of α_T and that this inhibition was overcome at high levels of rhodopsin. A potentially important outcome of the peptide 3/ α_T interaction is the facilitation of the activation of the α_T subunit. This was first demonstrated in fluorescence experiments where the binding of peptide 3 was shown to strongly promote the enhancement of the tryptophan emission of α_T that is elicited by the addition of NaF. Specifically, the EC_{50} for NaF was shifted from ~ 4 mM in the absence of peptide 3 to below 0.5 mM in the presence of peptide 3. Further verification that peptide 3 facilitated the ability of NaF to activate the α_T subunit was obtained from experiments measuring the α_T GDP/NaF-stimulated hydrolysis of cyclic GMP by the cyclic GMP phosphodiesterase.

INTRODUCTION

The phototransduction cascade of the mammalian rod cell [1,2] serves as an excellent model for signal transduction events that are mediated by three-component signalling systems, i.e. a cell surface receptor protein, a GTP-binding protein (G protein) and an effector enzyme or ion channel. The absorption of light by rhodopsin (a single-chain protein of $M_r \sim 40\,000$ [3]) promotes the coupling of the photoreceptor to a heterotrimeric GTP-binding protein, transducin. The M_r values of the α subunit (α_T), β subunit (β_T) and γ subunit (γ_T) of transducin are 39 000, 36 000 and 8 000 respectively [4,5]. The binding of rhodopsin to transducin triggers the dissociation of a tightly bound molecule of GDP, which then enables GTP to bind and to trigger an 'activating' conformational change within the α_T subunit. It is the activated α_T subunit that binds to the retinal effector enzyme, the cyclic GMP phosphodiesterase (a heterotrimeric protein with two larger subunits, $\alpha_{PDE} = \beta_{PDE}$ of $M_r \sim 85\,000$, and a third smaller subunit, γ_{PDE} of $M_r \sim 14\,000$ [6,7]). The activated α_T subunit stimulates the phosphodiesterase (PDE) to hydrolyse cyclic GMP; this stimulation continues until the GTPase activity of α_T converts the bound GTP back to GDP.

Rhodopsin is a member of the large class of G-protein-coupled membrane receptors that includes the α - and β -adrenergic receptors and the muscarinic acetylcholine receptors (see [8]). All of the members of this family have seven membrane-spanning helices which contain the hormone binding site or, in the case of rhodopsin, the Schiff base linkage for the chromophore, retinal. The G protein coupling site, by necessity, is located within the cytoplasmic domains of these receptors. The specific location of the receptor binding domains for the G protein α and $\beta\gamma$ subunits has been the subject of a great deal of interest, and

virtually every portion of the cytoplasmic domains of these receptors has in one study or another been implicated in receptor/G protein coupling [9–16]. We have been interested in mapping specific binding domains on rhodopsin for the individual α_T and $\beta\gamma_T$ subunit components of transducin. To do this we have synthesized a number of short peptides corresponding to the different putative cytoplasmic loops and the C-terminal tail of the protein, and then we have examined these peptides in a number of functional assays and fluorescence spectroscopic read-outs. In the studies outlined below we report that one of these synthetic peptides (KNPLGDDEASTTVS-amide), which represents amino acids 325–338 from the C-terminal tail of bovine opsin (see Figure 1 below), serves as a contact site for the α_T subunit. Based on our results, we propose that this binding interaction facilitates the occurrence of an activating conformational change within the α_T subunit.

MATERIALS AND METHODS

Purification of retinal proteins

Frozen dark-adapted bovine retina were purchased from Hormel (Austin, MN, U.S.A.). Rod outer segment (ROS) membranes were prepared as previously described [17,18] and either used directly in assays measuring cyclic GMP PDE activity or used for the further purification of rhodopsin and transducin. Rhodopsin was purified under dim red light by first washing the ROS membranes (five times) with hypotonic buffer (10 mM Hepes, pH 7.5, 6 mM $MgCl_2$, 1 mM dithiothreitol). The photoreceptor was solubilized with 20 mM CHAPS, 50 mM Tris/acetate (pH 7.0), 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 0.1 M NaCl [19,20], and then applied to a 10 ml concanavalin A–Sephacrose column and highly purified by elution with 0.1 M α -methylmannoside in

Abbreviations used: α_T , the α subunit of transducin; $\beta\gamma_T$, the $\beta\gamma$ subunit complex of transducin; GTP[S], guanosine 5'-[γ -thio]triphosphate; ROS, rod outer segment; PDE, phosphodiesterase.

* Present address: Alliance Pharmaceutical, P.O. Box 567, Old Mountain Road, Otisville, NY 10963, U.S.A.

the Tris acetate/CHAPS buffer. The purified rhodopsin (1–5 mg/ml) was stored in the dark at -70°C .

Holotransducin and the cyclic GMP PDE were purified by exposing the ROS membranes to room light during repeated washings with isotonic (10 mM Hepes, pH 7.5, 6 mM MgCl_2 , 1 mM dithiothreitol, 100 mM NaCl) and then hypotonic buffer. The cyclic GMP PDE is released into the hypotonic wash and can be further purified by hydroxyapatite chromatography [18], whereas the transducin remains associated with the ROS. The transducin was then released from the ROS by washing the membranes in hypotonic buffer supplemented with 0.1 mM GTP {or guanine 5'-[γ -thio]triphosphate (GTP[S])}. The supernatant fractions from three such washings were collected, and the α_T and $\beta\gamma_T$ subunit complexes were resolved by chromatography through Blue Sepharose [21]. When GTP was used to elute the transducin from the ROS, the purified α_T subunit contained tightly bound GDP (i.e. $\alpha_T\text{GDP}$), as a result of the hydrolysis of the GTP [22]. The purified subunits (typically 0.2–0.5 mg/ml) were stored at -70°C ; the α_T subunits were stored in 10 mM Hepes, pH 7.5, 6 mM MgCl_2 , 1 mM dithiothreitol, 25% glycerol and 0.5 M KCl, and the $\beta\gamma_T$ subunit complexes were stored in the same buffer but without KCl.

Reconstitution of rhodopsin/transducin interactions

Purified rhodopsin was inserted into phospholipid vesicles prepared from crude soybean phosphatidylcholine as previously described [23]. The interactions between the reconstituted rhodopsin and the purified α_T subunit were monitored by measuring the light-stimulated binding of [^{35}S]GTP[S] [23] or [γ - ^{32}P]GTP hydrolysis. The latter (GTPase) assays were performed by first mixing the purified $\alpha_T\text{GDP}$ species (typically ~ 15 pmol) and the purified $\beta\gamma_T$ (typically ~ 15 pmol) with phospholipid vesicles containing rhodopsin (~ 40 pmol) in a total volume of 50 μl . The assays then were initiated by the addition of assay buffer (final concentration 20 mM Hepes, pH 7.5, 2 mM MgCl_2 , 0.5–1 μM [γ - ^{32}P]GTP), with the incubations proceeding at room temperature for 10–20 min. The GTPase activities were determined by measuring the release of [^{32}P]P_i by an isobutanol/benzene extraction [24]. Controls were performed in which identical assay incubations were established in the absence of added α_T ; non-specific GTPase activity was typically $< 10\%$ of the total measured activities.

Fluorescence spectroscopy

The intrinsic tryptophan fluorescence of the α_T subunit was determined using an SLM 8000 spectrofluorimeter in the photon counting mode [20]. The samples of $\alpha_T\text{GDP}$ were diluted to a final concentration of ~ 500 pmol/ml in 20 mM Hepes, pH 7.5, 5 mM MgCl_2 , 1 mM dithiothreitol (final volume 0.15 ml), in the presence of NaF (0.5 mM or 5 mM) and/or peptide 3 (as indicated in the Results section) in 0.2 ml quartz cuvettes and the fluorescence emission at 330 nm (excitation 280 nm) was measured. The background fluorescence also was measured by scanning samples that lacked α_T and was subtracted (unless otherwise noted) from the total emission.

Phosphorylation of the α_T subunit by protein kinase C- β_1

The recombinant protein kinase C- β_1 protein, which was over-expressed in COS cells and then highly purified as described [25],

was the kind gift from Dr. Berta Strulovici (Tularik Research Institute, San Francisco, CA, U.S.A.). The phosphorylation of the α_T subunit was performed essentially as described in Phillips et al. [26]. Protein kinase C- β_1 (~ 0.03 ng) was added to an assay solution (50 μl) that contained 1 μg of phosphatidylserine, 1 μg of oleoylacetyl glycerol and the protein components (rhodopsin, α_T , $\beta\gamma_T$) or the synthetic peptides, as indicated in the Results section. The phosphorylation assays were initiated by the addition of [γ - ^{32}P]ATP (final concentration ~ 50 μM) and were performed for 30 min at 30°C .

Measurement of cyclic GMP PDE activity

Dark-adapted ROS membranes, or phospholipid vesicles containing hydroxyapatite-purified cyclic GMP PDE, were assayed for cyclic GMP hydrolysis using the proton release assay as initially described by Yee and Liebman [27]. The assays were typically performed at room temperature in a final volume of 0.3 ml in buffer containing 10 mM Hepes (pH 8.0), 60 mM KCl, 30 mM NaCl, 1 mM dithiothreitol and 5 mM MgCl_2 supplemented with different protein components as described in the legend to Figure 6. The assays were initiated by the addition of 5 mM (final) cyclic GMP, with the pH being recorded over the ensuing 60–90 s at 1 determination/s. The assays were stopped by the addition of 0.5 μmol of KOH, which serves to calibrate the buffering capacity of the assay. The PDE activity (nmol/s) was calculated as the ratio of the slope of the pH change (mV/s) and the buffering capacity of the medium (mV/nmol) [28].

Synthetic peptides

Synthetic peptides that represent the different putative cytoplasmic loops of bovine rhodopsin were synthesized by the Cornell Biotechnology Institute and then highly purified by h.p.l.c.

RESULTS

A primary aim of these studies was to determine whether a specific peptide representing one of the putative cytosolic loops of bovine rhodopsin was capable of influencing the guanine nucleotide exchange activity of retinal transducin, or some aspect of the activation of the α subunit of this G protein. We have examined a number of peptides in these studies including a peptide representing the putative second cytoplasmic loop (amino

Figure 1 Depiction of bovine rhodopsin and peptide sequences that represent putative cytoplasmic domains

Peptide 1, residues 141–151; peptide 2, residues 310–324; peptide 3, residues 325–338; peptide 4, residues 231–238; peptide 5, residues 239–252. The different peptides were synthesized by the Cornell Biotechnology Facility (see the Materials and methods section).

Figure 2 Effects of rhodopsin and rhodopsin-derived peptides on the protein kinase C-catalysed phosphorylation of the α_T subunit

The purified α_T subunit (80 pmol) was incubated in the presence of various amounts of rhodopsin (lane 2, 0.6 μ M; lane 3, 1.2 μ M; lane 4, 2.4 μ M), peptide 1 (750 μ M; lane 5), and peptide 3 (750 μ M; lane 6). The phosphorylation assays were initiated by the addition of [γ - 32 P]ATP to an assay mixture containing 0.032 ng of protein kinase C- β_1 (see the Materials and methods section). The assays were performed for 30 min at room temperature in room light and then the samples were electrophoresed on a 10% polyacrylamide/SDS gel. The gel was dried and autoradiographed using Kodak X-Omat film.

acid residues 141–151, designated peptide 1), peptides representing the two halves of the third cytoplasmic loop (amino acid residues 231–238, designated peptide 4; and residues 239–252, designated peptide 5), and peptides representing two portions of the C-terminal tail (amino acid residues 310–324, designated peptide 2; and residues 325–338, designated peptide 3) (see Figure 1). Thus far, we have found only one of these peptides (i.e. peptide 3) to be capable of influencing the functional activities of the α_T subunit.

Effects of rhodopsin and rhodopsin peptides on the phosphorylation of the α_T subunit by the protein kinase C- β_1 isoform

We [26] and others [29] have reported that protein kinase C is capable of phosphorylating the α_T subunit *in vitro* and that the interactions of the α_T subunit with rhodopsin stimulate the protein kinase C-catalysed phosphorylation of α_T [26]. An example is shown in Figure 2. Increasing amounts of light-activated rhodopsin (over a range of 0.6–2.4 μ M) caused an approx. 3-fold increase in the protein kinase C- β_1 -catalysed phosphorylation of the α_T subunit. Although not shown here, in some cases a weaker protein kinase C-mediated phosphorylation of rhodopsin was also detected (as represented by a phosphorylated band that migrates just below the phosphorylated α_T [26]). We examined whether any of the rhodopsin-derived peptides was capable of eliciting a similar 'stimulatory effect' on the phosphorylation of α_T by protein kinase C- β_1 . As shown in Figure 2, peptide 3 was capable of stimulating the protein kinase C- β_1 -catalysed phosphorylation of the α_T subunit. We did not find this stimulatory effect with any of the other rhodopsin peptides that we synthesized (see Figure 1). In fact, the one other peptide that we have found to have an effect (i.e. peptide 1) actually caused a slight inhibition of phosphorylation. Overall, these data provided us with the first line of evidence that the C-terminal peptide of rhodopsin (peptide 3) is capable of binding directly to the α_T subunit.

Effects of peptide 3 on the guanine nucleotide exchange activity of the α_T subunit

Given that peptide 3 appeared to be capable of mimicking rhodopsin by increasing the susceptibility of α_T to phosphorylation by protein kinase C- β_1 (presumably via the induction of a conformational change within the α_T subunit), we next examined

whether peptide 3 is able to stimulate the guanine nucleotide exchange reaction of either the purified holotransducin molecule or the purified α_T GDP subunit. This was done by measuring [35 S]GTP[S] binding (which occurs as an outcome of GDP-[35 S]GTP[S] exchange) and [γ - 32 P]GTP hydrolysis (i.e. the outcome of GDP-[γ - 32 P]GTP exchange). In most experiments the results were negative, i.e. peptide 3 (when examined at concentrations up to 1 mM) did not elicit a significant stimulation of either of these activities, when the α_T subunit was assayed in both the presence and the absence of the $\beta\gamma_T$ complex. However, in some experiments peptide 3 (at concentrations in the range 0.1–1 mM) was able to stimulate [35 S]GTP[S] binding to the α_T subunit (by as much as 2-fold; results not shown). The peptide 3-stimulated [35 S]GTP[S] binding was only 10–20 % of the binding stimulated by light-activated rhodopsin, which indicated that peptide 3 was not able to fully substitute for rhodopsin and effectively catalyse the guanine nucleotide exchange activity of the α_T subunit. Thus far, we have not found any other peptide to be capable of influencing the guanine nucleotide exchange activity of α_T .

We next examined whether peptide 3 could compete with light-activated rhodopsin for binding to transducin and thereby interfere with the rhodopsin-stimulated GTP-binding or GTPase activities of α_T . The results presented in Figure 3 indicate that peptide 3 was capable of eliciting a dose-dependent inhibition ($IC_{50} \sim 0.5$ mM) of the [γ - 32 P]GTP hydrolysis associated with α_T in phospholipid vesicle systems where the concentration of rhodopsin was ~ 8 nM. These results also show that the ability of peptide 3 to inhibit the rhodopsin-stimulated GTPase activity was dependent on the concentration of rhodopsin present in the assay incubation. For example, at a 10-fold higher concentration of rhodopsin, inhibition by peptide 3 was significantly reduced (such that the IC_{50} for peptide 3 was increased to greater than 1 mM). In these competition experiments, we have not found the $\beta\gamma_T$ subunit complex to be capable of reversing the peptide 3-mediated inhibition of rhodopsin-stimulated GTPase activity (results not shown). Thus these results are consistent with a model where peptide 3 and rhodopsin compete for a common binding site on the α_T subunit (and not the $\beta\gamma_T$ subunit complex) of transducin.

Fluorescence spectroscopic read-outs for peptide 3/transducin interactions

Previous studies have shown that the exchange of GDP for GTP (or GTP[S]) on the α_T subunit is accompanied by an enhancement of the α_T tryptophan fluorescence [20,23]. The addition of peptide 3 to α_T , at concentrations which enhanced the phosphorylation of the α_T subunit by protein kinase C- β_1 (Figure 2) and inhibited the rhodopsin-stimulated GTPase activity (Figure 3), had no significant effect on the tryptophan emission of α_T either in the presence or in the absence of added guanine nucleotide (results not shown). This was consistent with our findings that peptide 3 was incapable of mimicking rhodopsin by causing a marked stimulation of the guanine nucleotide exchange activity of α_T . While these results indicated that peptide 3 did not trigger the first step necessary for the activation of the α_T subunit (i.e. GDP dissociation), we questioned whether peptide 3 might be capable of influencing the conformational change in the α_T subunit that occurs as an outcome of activation and primes α_T for binding to its effector protein (the cyclic GMP PDE). To examine this, we took advantage of the fact that G protein α subunits are able to be activated by NaF, in a manner independent of guanine nucleotide exchange. In this case, fluoride is thought to complex with the bound GDP of the α subunit and mimic the effects

Figure 3 Inhibition of the rhodopsin-dependent GTPase activity of α_1 GDP by peptide 3

Purified rhodopsin was reconstituted into phosphatidylcholine vesicles as described in the Materials and methods section. The GTPase activity of the α_1 GDP species (15 pmol) was measured in an assay mixture containing $\beta\gamma_T$ (15 pmol), rhodopsin-containing lipid vesicles [4 pmol ($\square$) or 0.4 pmol ($\blacksquare$)], and peptide 3 at the concentrations indicated. The amount of [32 P] released, as an outcome of [γ - 32 P]GTP hydrolysis, was measured for 20 min at room temperature. The error bars represent the ranges of duplicate determinations.

Figure 4 Dose-response curves for the NaF-induced enhancement of α_1 GDP tryptophan fluorescence

The α_1 GDP fluorescence (excitation = 280 nm, emission = 335 nm) was measured by diluting 50 pmol of the purified α_1 GDP with 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol plus the indicated levels of NaF in the absence ($\square$) and in the presence ($\blacksquare$) of 500 μ M peptide 3. All measurements were corrected for background fluorescence by scanning identical incubations that lacked α_1 GDP and subtracting these values from the total.

elicited by GTP. The activation of G protein α subunits by millimolar concentrations of NaF has been suggested to be the outcome of the formation of an aluminum fluoride (AlF_4^-) complex (from contaminating aluminium) [30]. However, it was reported recently that millimolar concentrations of fluoride, in the presence of Mg^{2+} , will alone (i.e. in the absence of aluminium) induce an active state within G protein α subunits that is structurally analogous to that induced by AlF_4^- or by GTP [31]. For simplicity, we refer to this as a NaF-induced activation throughout the text. As shown in Figure 4, we found that peptide 3 significantly influenced the ability of the α_T GDP complex to undergo a NaF-induced activating conformational change, as

Figure 5 Dose-response curve for the promotion by peptide 3 of the NaF-induced enhancement of α_1 GDP fluorescence

The α_1 GDP species (50 pmol) was diluted to 0.15 ml with 20 mM Hepes/ Na^+ , pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, plus 0.5 mM NaF and the indicated amounts of peptide 3. These mixtures were incubated for 5 min at 23 $^{\circ}C$ and then the fluorescence emission was measured (excitation = 280 nm, emission = 335 nm). All measurements were corrected for background fluorescence by measuring the emission of an identical mixture that lacked α_1 GDP and then subtracting these measurements from the total fluorescence (measured in the presence of α_1 GDP). The results shown are the means of two experiments (the error bars represent the ranges for the two determinations).

shown by an enhancement of the α_T tryptophan fluorescence. Specifically, in the absence of peptide 3, NaF promoted the enhancement of the tryptophan emission of the α_T subunit with an EC_{50} of approx. 5 mM, whereas in the presence of 0.75 mM peptide 3, significantly lower concentrations of NaF were able to stimulate the enhancement of the tryptophan fluorescence of α_T . These effects were absolutely specific for peptide 3 and were not elicited by any of the other synthetic peptides depicted in Figure 1.

Figure 5 shows that the ability of peptide 3 to promote the enhancement of the tryptophan fluorescence of the α_T GDP complex (in the presence of 0.5 mM NaF) is dose-dependent. The half-maximal fluorescence enhancement occurred at concentrations of peptide 3 that were similar to the concentrations that caused an inhibition of the rhodopsin-stimulated GTPase activities (i.e. $EC_{50} \sim 0.2$ –0.4 mM).

It has been previously demonstrated that millimolar concentrations of Mg^{2+} are necessary for the maximal activation of G protein α subunits by NaF, i.e. in the presence of excess EDTA the NaF-induced activation is eliminated [32]. We questioned whether the promotion by peptide 3 of the NaF-induced enhancement of α_T tryptophan fluorescence might reflect an increase in the affinity of the α_T subunit for Mg^{2+} and/or the outcome of some type of peptide 3/bivalent metal ion complex. However, this does not appear to be the case. The Mg^{2+} -dependencies for the NaF-induced enhancement of α_T emission were essentially identical in the presence and absence of saturating levels of peptide 3 (results not shown).

Activation of the retinal effector enzyme, cyclic GMP PDE, by peptide 3 and NaF

The results of the fluorescence studies presented in Figures 4 and 5 suggested that peptide 3 facilitated the (NaF-induced) activation of the α_T subunit. We therefore set out to examine this directly by assaying cyclic GMP PDE activity (i.e. the biological

Figure 6 Promotion by peptide 3 of NaF-stimulated PDE activity

(a) Dark-adapted ROS membranes (prepared as described in the Materials and methods section) were assayed for endogenous PDE activity alone, in the presence of 50 pmol of α_T GDP, 50 pmol of α_T GDP plus 5 mM NaF or 50 pmol of α_T GDP plus 0.5 mM NaF and 500 μ M peptide 3. The bars labelled 'control' represent assays performed in the absence of (5 mM) NaF. Identical control activities were obtained in the presence of 0.5 mM NaF (in the absence of peptide 3). The assays were initiated by the addition of 5 mM (final concentration) cyclic GMP and the data acquisition was continued for approx. 100 s. The initial rate of cyclic GMP hydrolysis was determined as described in the Experimental section. Each bar represents the means of duplicates (error bars show ranges). (b) Experiments were performed as described in (a) except that ~ 4 nM hydroxyapatite-purified PDE (see the Materials and methods section) was substituted for the ROS. In experiments where peptide 3 was not included, the NaF concentration was 5 mM. In experiments where peptide 3 was included, the NaF concentration was 0.5 mM. The basal (control) levels of cyclic GMP hydrolysis (solid bar) were not changed by the addition of 0.5 mM NaF alone or by the addition of 500 μ M peptide 3 alone. In some cases, 40 pmol of the AS/7 antibody was included in the assay mixture.

target of the activated α_T subunit) in the presence and absence of peptide 3. In control experiments we first established that peptide 3 had no effect on the basal activity of the cyclic GMP PDE (i.e. in the absence of an activated α_T subunit) nor on the catalytic activity of trypsin-activated PDE (results not shown). As shown in Figure 6(a), the addition of NaF (5 mM) to ROS that contained both (endogenous) transducin and PDE resulted in stimulation of cyclic GMP hydrolysis. In the absence of peptide 3, 0.5 mM NaF had no effect on the basal cyclic GMP PDE activity; however, in the presence of peptide 3, the exposure of ROS to 0.5 mM NaF resulted in a stimulated PDE activity that was comparable with, and often exceeded, that elicited by 5 mM NaF. When exogenous α_T GDP was added to the ROS, the total PDE activity was increased. This indicated that the stimulation of the PDE activity was dependent on the total amount of

α_T GDP present in the membranes and that the endogenous level of α_T represented a sub-saturating concentration (relative to the PDE). When assaying the PDE activity under optimal conditions (i.e. upon the addition of purified α_T GDP), we again found that the extents of activation of the PDE were greatest when adding (0.5 mM) NaF together with peptide 3; this was even the case when using NaF concentrations as high as 20 mM (in the absence of peptide 3; results not shown).

The ability of the α_T GDP complex, incubated either with a high concentration of NaF (5 mM) or with a low concentration of NaF (0.5 mM) together with peptide 3, to stimulate hydroxyapatite-purified PDE also was examined (Figure 6b). The results of these studies were similar to those obtained when using the ROS preparations. Peptide 3 enabled sub-saturating concentrations of NaF (0.5 mM) to activate the α_T GDP complex and increased the extent of stimulation of PDE activity.

Previously we reported that an antibody raised against the C-terminal decapeptide of the α_T subunit (designated AS/7) was capable of potentiating the stimulation of the PDE by an activated α_T GTP[S] complex [18,28]. This promotion by AS/7 was specific for the bivalent form of the antibody and was not obtained when using monovalent Fab fragments; this led to the suggestion that AS/7 promoted the α_T GTP[S] stimulation of the effector by simultaneously delivering two activated α_T subunits to the effector enzyme [18]. The results presented in Figure 6(b) show that the AS/7 antibody was able to potentiate the ability of the NaF-activated α_T subunit to stimulate PDE activity. A similar potentiation also was observed for α_T subunits activated by (0.5 mM) NaF together with peptide 3. Thus the binding of peptide 3 to the α_T subunit does not interfere with the binding of AS/7 to the C-terminus of α_T .

DISCUSSION

A key area of interest concerning the structure and function of G-protein-coupled receptors has been the identification of the specific intracellular domains of the receptors that are involved in functionally coupling to the G proteins. One approach that has been taken to identify the binding domains for holotransducin on the rhodopsin molecule involves the use of short synthetic peptides that represent different regions of the putative cytosolic domains of the receptor. In one series of studies [13], a number of peptides from the C-terminal tail of rhodopsin were examined for their ability to inhibit the rhodopsin-stimulated guanine nucleotide exchange activity of transducin (as monitored by the inhibition of rhodopsin-stimulated GTPase activity). The conclusion from these studies was that the C-terminal tail of rhodopsin, in particular amino acid residues 317–339, was an important part of the transducin binding domain. However, in another study, it was suggested that multiple regions were involved in binding the retinal G protein [15]. Specifically, it was shown that peptides from the second and third intracellular loops, as well as a peptide representing amino acids 310–323 from the fourth (C-terminal) intracellular loop, were able to inhibit (competitively) the binding of transducin to light-activated rhodopsin as measured by a stabilization of the *meta* II conformation of rhodopsin. It was further suggested that combinations of these peptides acted synergistically to inhibit the association of rhodopsin and transducin.

We have set out to extend these findings by using a variety of assays to determine regions within the cytosolic domain of rhodopsin that bind either to the α_T or $\beta\gamma_T$ subunit complex and/or influence some aspect of the GTP binding/GTPase cycle of α_T . Recently we have suggested that a peptide representing amino acids 310–324 from the putative fourth cytosolic loop of

rhodopsin (designated peptide 2 in Figure 1) might represent the binding site for the $\beta\gamma_T$ complex, since this peptide specifically elicited changes in the fluorescence of a 2-(4-maleimidylanilino)-naphthalene-6-sulphonic acid (MIANS)-labelled $\beta\gamma_T$ [26,33]. In the present study, we have identified another peptide from the C-terminal tail region of rhodopsin (amino acid residues 325–338, designated peptide 3) that appears to bind specifically to the α_T subunit. The initial suggestion came from studies examining the ability of different rhodopsin-derived peptides to bind to the α_T subunit and increase its phosphorylation by protein kinase C- β_1 [26]. The rationale for these studies was based on our earlier finding that the binding of rhodopsin to α_T enhanced the ability of α_T to serve as a substrate for protein kinase C, presumably via a rhodopsin-promoted conformational change in this subunit [26]. Of the various rhodopsin peptides examined (Figure 1), only peptide 3 showed any ability to mimic the actions of rhodopsin in this phosphorylation assay. One other peptide, which represented the putative second cytosolic loop of rhodopsin (peptide 1), showed an effect; however, this peptide caused an inhibition rather than a stimulation of α_T phosphorylation. It is interesting that peptide 1 also appeared to be capable of inhibiting rhodopsin-stimulated [^{35}S]GTP[S] binding and [γ - ^{32}P]GTP hydrolytic activity (results not shown), which provided further evidence for an interaction between this peptide and the α_T subunit. At the present time, we do not know the reason for the inhibitory effect elicited by peptide 1 on the phosphorylation of α_T by protein kinase C; however, one possibility is that peptide 1 binds to α_T at a site that overlaps the protein kinase C phosphorylation site (i.e. at the N-terminus of the α_T subunit [26]).

Although peptide 3 was able to mimic the actions of rhodopsin and promote the phosphorylation of the α_T subunit by protein kinase C- β_1 , unlike rhodopsin peptide 3 was not able to cause a marked stimulation of the guanine nucleotide exchange reaction. At best, we observed only a 2-fold stimulation by peptide 3 of the binding of [^{35}S]GTP[S] to the α_T subunit; thus peptide 3 was typically 10-fold less effective than rhodopsin in stimulating nucleotide exchange. Peptide 3 was able to inhibit rhodopsin-stimulated GTP binding (results not shown) and GTPase activity (Figure 3), as would be expected if both rhodopsin and peptide 3 competed for the free α_T subunit, and this inhibition was prevented at high levels of rhodopsin, consistent with the hypothesis that peptide 3 and rhodopsin share a common domain on the α_T subunit. Overall, these results suggest that while peptide 3 could bind to α_T and induce a conformational change within this subunit which apparently shared some similarity with the conformational change(s) elicited by rhodopsin (as indicated from the phosphorylation studies), the peptide 3/ α_T interaction did not significantly influence the rate of dissociation of GDP from α_T .

The results of our fluorescence studies suggest that the interaction between peptide 3 and α_T may influence a step that normally occurs subsequent to guanine nucleotide exchange, namely the conformational change that accompanies the activation of the α_T subunit. This conformational change presumably primes α_T for interacting with the downstream effector target (the cyclic GMP PDE). Although peptide 3 showed no detectable effects on the intrinsic tryptophan fluorescence of the α_T subunit, in the presence or absence of added guanine nucleotide, it significantly altered the dose response for a NaF-induced enhancement of tryptophan fluorescence. Specifically, in the presence of peptide 3, the NaF-stimulated enhancement of the α_T tryptophan fluorescence was maximal at 0.5 mM NaF, whereas in the absence of peptide 3 no enhancement of the tryptophan fluorescence was observed at that concentration of NaF (see

Figure 4), and only half-maximal enhancement was achieved at ~4 mM NaF. The peptide 3-mediated effects on the NaF-induced fluorescence changes occurred over a concentration range of the peptide that matched the concentration range for the peptide 3-mediated inhibition of rhodopsin/holotransducin coupling, consistent with the notion that a common peptide 3/ α_T binding interaction was responsible for these different effects.

The data obtained from PDE assays support the suggestion that the binding of peptide 3 to α_T enhances the ability of this G protein subunit to undergo an 'activating' conformational change. In the presence of peptide 3, 0.5 mM NaF elicited a significant (α_T GDP-mediated) stimulation of the cyclic GMP PDE, both in ROS that contained endogenous PDE and in reconstituted systems containing the partially purified effector enzyme. In the absence of peptide 3, no NaF-promoted stimulation was observed until levels of 2–5 mM NaF were used. One possible explanation for these results is that the binding of peptide 3 to the α_T subunit has a direct influence on the rate of dissociation of the fluoride ion (i.e. peptide 3 could increase the affinity of α_T for fluoride by reducing the rate of dissociation of fluoride from the subunit). While it is not possible for us to definitively rule out this possibility, we feel that there are two observations that support a model where a peptide 3-induced conformational change is involved in facilitating or stabilizing the activation of the α_T subunit. Specifically, the phosphorylation studies support the idea that peptide 3 induces a conformational change within the α_T subunit (that increases the ability of this subunit to serve as a substrate for phosphorylation), and the PDE assays consistently show that the levels of cyclic GMP hydrolysis achieved in the presence of peptide 3 and NaF were greater than those achieved with NaF alone (even at high concentrations of NaF, i.e. 20 mM).

At the present time, we do not know the location of the peptide 3 binding site on the α_T subunit. However, given that peptide 3 stimulates, and does not inhibit, the phosphorylation of α_T by protein kinase C, it appears unlikely that peptide 3 binds to the extreme N-terminal domain of α_T , since this region contains the site of phosphorylation by protein kinase C (most likely serine-12) [34]. It also seems unlikely that peptide 3 binds to the extreme C-terminal domain (which is felt to contain at least a portion of the rhodopsin binding domain [28]), since peptide 3 does not interfere with the ability of AS/7 (an anti- α_T antibody raised against the C-terminal decapeptide of α_T) to potentiate the stimulation (by an activated α_T species) of the PDE. In the future, it will be interesting to determine the site of interaction of the α_T subunit with peptide 3, as well as determining whether other rhodopsin peptides, in combination with peptide 3, can potentially stimulate the GDP–GTP exchange reaction in the α_T subunit.

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