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Effect of detergents and lipids on transducin photoactivation by rhodopsin

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Rhodopsin samples, isolated using four different extraction procedures, were used to investigate the photodependent activation of the GTPase activity of transducin. A complete inhibition of transducin light-dependent GTP hydrolytic activity was observed when rhodopsin purified in the presence of 1% digitonin, following rod outer segment (ROS) solubilization with 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), was used for its activation [0 pmol of inorganic phosphate (P_i) released/min/pmol of rhodopsin]. Rhodopsin, isolated in the presence of 1% digitonin following ROS solubilization with 1% digitonin, was capable of stimulating slightly transducin GTPase activity, with an initial rate of 1 pmol of GTP hydrolyzed/min/pmol of rhodopsin. However, rhodopsin purified in the presence of 0.2% n-dodecyl-β-D-maltoside (DM), following ROS solubilization with either 1% CHAPS or 1% DM, stimulated the enzymatic activity of transducin in a light-dependent manner, with an initial rate of 5 pmol of P_i released/min/pmol of rhodopsin. Addition of 0.075% egg phosphatidylcholine (PC) to the four different solubilized rhodopsin samples significantly enhanced light-stimulated GTP hydrolysis by transducin, with initial rates increasing from 0 to 1, 1 to 2, and 5 to 30 pmol of P_i released/min/pmol of rhodopsin, respectively. Furthermore, DM-solubilized rhodopsin induced the hydrolysis of the maximum amount of GTP by transducin at 0.0075% PC, while digitonin-solubilized rhodopsin only stimulated the GTPase activity of transducin to a similar value, when the amount of the photoreceptor protein was increased 4-fold and 0.15% PC was added to the assay mixture. These results suggest that the effective photoactivation of transducin by rhodopsin requires phospholipids, which seem to be differentially eliminated with the detergent extraction procedure utilized during ROS membranes solubilization and photopigment isolation.

Key terms: *detergent solubilization, G-proteins, G-protein coupled receptors, lipids requirement, membrane proteins, signal transduction, visual process.*

ABBREVIATIONS

CHAPS, 3-[(3-cholamidopropyl) dimethylammonio] -1-propane-sulfonate
DM, n-dodecyl-β-D-maltoside
EDTA, ethylenediaminetetraacetic acid
Emulphogene, polyoxyethylene-10 tridecylether
G-protein, guanine nucleotide-binding regulatory protein
Meta I, metarhodopsin I
Meta II, metarhodopsin II

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Octylglucoside, N-octyl-β-D-glucopyranoside
PC, egg phosphatidylcholine
PEI, polyethyleneimine
PMSF, phenylmethylsulfonylfluoride
ROS, rod outer segment
SDS, sodium dodecyl sulphate

INTRODUCTION

The dim-light photoreceptor rhodopsin is a prototypical member of the superfamily of guanine nucleotide-binding protein (G-protein) coupled receptors sharing the seven-transmembrane-helix structural motif, which regulates a variety of sensory, hormonal and neural responses. Rhodopsin is composed of the apoprotein opsin, a single polypeptide chain of 348 amino acids (Ovchinnikov *et al*, 1982; Hargrave *et al*, 1983), and a covalently linked 11-*cis* retinal chromophore. The chromophore is bound to the ε-amino group of Lys_{296}, located in the seventh helix via a protonated Schiff base linkage (Dratz & Hargrave, 1983; Wald, 1968). In the ground state this charge is stabilized by the counter-ion Glu_{113} located in the third helix (Sakmar *et al*, 1989; Zhukovsky & Oprian, 1989; Nathans, 1990). Light-induced isomerization of the chromophore to the all-*trans* conformation results in the formation of a series of photointermediates (Hubbard & Kropf, 1958), which have been identified by their characteristic absorption spectra (Matthews *et al*, 1963; Yoshizawa & Wald, 1963; Busch *et al*, 1972). One of the photoproducts, metarhodopsin II (Meta II), is the active conformation of rhodopsin (Emeis *et al*, 1982; Kibelbek *et al*, 1991) which binds and activates transducin, the visual G-protein, triggering a signal transduction cascade that leads to the closing of cGMP-dependent cationic channels in the rod cell plasma membrane. The resulting hyperpolarization is then transmitted from the synaptic end of the photoreceptor cell to connected nerve cells (Stryer, 1986).

The light-dependent rhodopsin activity is transient and decays rapidly under *in vivo* conditions because of the action of two other proteins in the rod outer segment (ROS): rhodopsin kinase and arrestin (Bennett & Sitaramayya, 1988; Palczewski & Benovic, 1991; Palczewski *et al*, 1992). Rhodopsin kinase phosphorylates Meta II at multiple sites on the carboxy-terminal tail of the protein (Ohguro *et al*, 1993; Palczewski *et al*, 1991). The phosphorylated protein then binds arrestin in a reaction that inhibits competitively the interaction between rhodopsin and transducin, bringing the activation reaction to an intermediate halt (Schleicher *et al*, 1989; Wilden *et al*, 1986). Once the activity of Meta II is quenched by phosphorylation and the binding of arrestin, the dark state of rhodopsin, with 11-*cis* retinal bound to the protein, is regenerated by a series of reactions that involve hydrolysis of the Schiff base linkage, dissociation of all-*trans*-retinal from the protein, rebinding of 11-*cis* retinal, dissociation of arrestin, and finally, dephosphorylation by a rod cell specific phosphatase 2A (Palczewski & Benovic, 1991). The newly regenerated rhodopsin is now poised for the absorption of a second photon of light to initiate the enzymatic cascade once again.

Rhodopsin is an integral protein in the ROS membrane. X-ray diffraction data (Blaise, 1972; Blaurock & Wilkins, 1972; Chabre, 1975) and freeze fracture electron microscopy experiments (Chen & Hubbell, 1973) suggest that a major part of the protein molecule is in contact with the hydrophobic interior of the membrane. On the basis of sequence comparison of 204 members of the G-coupled receptor family, Baldwin has proposed a packing arrangement of the transmembrane helices in rhodopsin (Baldwin, 1993). Recently, low resolution structural models of frog and bovine rhodopsin have been derived from cryoelectron microscopy (Schertler *et al*, 1993) and electron diffraction data from two-dimensional crystals (Unger & Schertler, 1995; Schertler & Hargrave, 1995). Four transmembrane helices probably slant as they cross the membrane, while the remaining three helices are presumed to be oriented perpendicular to the plane of the membrane (Bourne, 1997). The model is generally consistent with the

packing arrangement suggested by Baldwin (1993). Thus, the environment provided by the lipid matrix of the disk membrane must influence directly the functional properties of rhodopsin.

Optimal choice of the type and concentration of detergent, to achieve the successful solubilization of functional integral membrane proteins, is partially still a subject of trial and error. Also, the manner in which surfactants affect the amount and type of native lipids that remain associated, or are concomitantly extracted with the membrane proteins during the solubilization process, requires further investigation. Retinal ROS provide an interesting membrane model for these studies, since the hydrophobic media of choice (native or washed ROS membranes, detergents, detergent-lipid mixtures, recombinant lipid bilayers, *etc*) might influence the stability of the reactive rhodopsin intermediates produced upon illumination, and/or the light-dependent reactions of rhodopsin (photoactivation of transducin, light-dependent phosphorylation by rhodopsin kinase, arrestin binding, *etc*). Here, I analyzed the effect of different detergents, and the presence of egg phosphatidylcholine, on transducin photoactivation by rhodopsin.

METHODS

Materials.

Bovine eyes were obtained from the nearest slaughterhouse (Matadero Caracas, C.A.). Retinae were extracted in the dark, under red light, and were maintained frozen at -70°C. [γ-^{32}P] GTP (25-33 Ci/mmol) was purchased from Amersham, Dupont-NEN Research Products, or ICN Biomedicals. Materials were obtained from the following suppliers: diethylaminoethylcellulose DE 52, Whatman; OptiPhase Hisafe II (scintillation liquid), LKB; GTP, Pharmacia; 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), digitonin, egg phosphatidylcholine (PC, 99%), alkaline phosphatase, 1-amino-2-naphthol-4-sulfonic acid, molybdic acid, phenylmethylsulfonylfluoride (PMSF), Sigma; n-dodecyl-β-D-maltoside (DM), Anatrace; Polygram Cel 300 PEI pre-coated plastic sheets for thin layer chromatography or PEI-cellulose (0.1 mm cellulose MN 300 polyethyleneimine impregnated), Macherey-Nagel + Co. 1D4-Sepharose and 1'-18' competing synthetic peptide (corresponding to the carboxyl terminal eighteen amino acids of rhodopsin, sequence = $^{+}H_3N$-Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala-COO^{-}) were the generous gift of Dr Barry Knox, State University of New York at Syracuse, USA. All the other reagents were of analytical grade.

Preparation of rod outer segments.

Rod outer segments (ROS) were isolated from frozen bovine retinae by flotation and subsequent centrifugation on discontinuous sucrose gradients (Papermaster & Dryer, 1974). The whole procedure was performed in the dark or in dim red light, on ice, and all sucrose solutions were prepared in Buffer I [70 mM potassium phosphate (pH 6.8), 1 mM magnesium acetate, 5 mM β-mercaptoethanol, and 0.1 mM PMSF]. Briefly, 100 retinae were thawed and extracted with 30% sucrose. The homogenate was centrifuged at 2,987 g for 6 min, at 4°C, and the supernatant containing the crude ROS was decanted into a flask. The resulting pellet was re-extracted as described above. After centrifugation, the second supernatant was decanted into the same flask, and the solution was diluted to 15% sucrose by slow addition of Buffer I. The crude ROS were pelleted at 11,950 g for 10 min, at 4°C. The pellet was suspended in 15% sucrose, divided into centrifuge tubes, underlayed with 0.64 M sucrose at the base of the tubes using a long needle, and centrifuged for 10 min at 11,950 g. Then, the pellet containing the ROS was suspended in 0.64 M sucrose by manual homogenization with a pestle and by passage through a N° 23 gauge needle.

Six three-step sucrose gradients were prepared in Beckman ultra-clear centrifuge tubes (25 x 89 mm) in the following manner: 9 mL of 0.84 M sucrose were

initially added to the centrifuge tubes; and this solution was slowly underlayed, first with 9 mL of a 1.00 M sucrose solution and then with 9 mL of a 1.20 M sucrose solution. Finally, 8-9 mL of the crude ROS suspended in 0.64 M sucrose were carefully overlayed on the top of each gradient. The tubes were balanced and centrifuged at 140,000 g, for 30 min, at 4°C, without the brake, in a Beckman L3-50 ultracentrifuge, using a SW 28 rotor. ROS usually banded at the 0.84 M sucrose/1.00 M sucrose interface, and were recovered by puncturing the tube wall at a level just beneath this layer with a N° 18 needle and aspirating with a clear 10 mL syringe. The ROS membranes were then diluted with Buffer I to yield a concentration of 2.5-3.0 mg/mL of rhodopsin (average absorbance ratio $A_{280\ nm}/A_{500\ nm}$ = 2.2-2.5), and kept frozen at -70°C until later use. Rhodopsin concentration was calculated from its UV/visible absorption spectra, using the molar extinction coefficient of the protein (40,700 $M^{-1}cm^{-1}$ at 500 nm) (Wald & Brown, 1953-1954).

Purification of rhodopsin by immunoaffinity chromatography.

Four different treatments were performed for the solubilization and purification of the photopigment, which are summarized in Table I. Dark-purified ROS (40 µL) were solubilized with 1% CHAPS, 1% DM, or 1% digitonin in Buffer II [50 mM Tris-HCl (pH 6.8), 100 mM NaCl and 0.1 mM $CaCl_2$], final volume = 5 mL, by agitation on a rotating wheel for 30 min at 4°C. The samples were centrifuged at 100,000 g, for 30 min at 4°C, and the supernatants were transferred to different tubes. Rhodopsin was purified by batchwise immunoaffinity chromatography on 1D4-Sepharose (Oprian *et al*, 1987). This resin contains the monoclonal antibody 1D4 covalently bound, which recognizes eight amino acids (1'-8') at the carboxy-terminus of rhodopsin. The interaction between the monoclonal antibody 1D4 and rhodopsin can be completely blocked by a synthetic peptide containing the last eighteen amino acids of the protein (1'-18') (MacKenzie *et*

Table I

Purification of rhodopsin samples by immunoaffinity chromatography on 1D4-Sepharose.

	Sample #1	Sample #2	Sample #3	Sample #4
Step 1: ROS solubilization[a]	1% CHAPS	1% digitonin	1% CHAPS	1% DM
Step 2: Column wash while rhodopsin was bound to 1D4-Sepharose affinity resin[a]	1% CHAPS	1% digitonin	1% CHAPS	0.2% DM
Step 3: Quick column wash prior to elution of rhodopsin[a]	1% digitonin	—	0.2% DM	—
Step 4: Elution of rhodopsin[a,b]	1% digitonin	1% digitonin	0.2% DM	0.2% DM
Rhodopsin spectral ratio ($A_{280\ nm}/A_{500\ nm}$)	1.76	1.82	1.90	1.77
Rhodopsin concentration (pmol/µL)[c]	0.8	0.9	1.2	1.2
Total protein (µg)[d]	70	79	108	102

Four different extraction procedures performed to purify rhodopsin: samples #1-#4. All detergent solutions were prepared in Buffer II (See Methods). [a] Concentration of detergent in solution used during specific purification step. [b] Rhodopsin eluted by incubating affinity matrix with 50 µL 1'-18' competing synthetic peptide in a solution of Buffer II containing described concentration of detergent. [c] Concentration of rhodopsin calculated using molar extinction coefficient of protein at 500 nm (Wald & Brown, 1953-54). [d] Amount of protein measured as reported by Bradford (1976).

al, 1984). Briefly, an aliquot (300 µL) of a 1D4-Sepharose suspended resin (1:1 with buffer) was added to each sample, and incubated on the rotating wheel for 4 hours at 4°C. Rhodopsin bound to the affinity matrix was washed three times with 1% CHAPS, 0.2% DM, or 1% digitonin in Buffer II. Two quick washes with either 1% digitonin or 0.2% DM in Buffer II, were performed in cases where a different detergent was to be used in the elution of the protein. Rhodopsin was eluted by incubating the affinity matrix with 50 µL 1'-18' competing synthetic peptide in a solution of Buffer II containing 1% digitonin or 0.2% DM. The eluates were centrifuged at 100,000 g and the rhodopsin samples were stored at -70°C. As seen in Table I, Sample #1 was solubilized in the presence of 1% CHAPS and eluted from the column with buffer containing 1% digitonin; Sample #2 was solubilized and eluted with buffer containing 1% digitonin, Sample #3 was solubilized with buffer containing 1% CHAPS and eluted in the presence of 0.2% DM; and Sample #4 was solubilized in the presence of 1% DM and eluted with buffer containing 0.2% DM. In all cases, the complete purification procedure was carried out in the dark or in dim red light.

Transducin isolation.

Transducin was isolated from ROS membranes prepared under room light, at 4°C, following the affinity binding procedure carried out by Kühn (1980). GTP (40-100 µL) was used to elute transducin from the washed illuminated ROS membranes, and transducin was further purified to homogeneity by anion-exchange chromatography on diethylaminoethyl cellulose DE 52, as described elsewhere (Bubis & Khorana, 1990; Bubis *et al*, 1993; Bubis *et al*, 1995). Aliquots of purified transducin were analyzed by SDS-polyacrylamide gel electrophoresis. The three components of transducin (T_α, T_β, and T_γ) migrating at apparent molecular weights of 40,000, 36,000, and 8,000, respectively, were detected by Coomassie blue staining (Data not shown). Transducin was stored at -20°C in storage buffer [20 mM Tris-HCl (pH 7.4), 5 mM magnesium acetate, 100 mM NaCl, 10 mM β-mercaptoethanol, 50% glycerol].

Determination of the integrity of [γ-^{32}P] GTP.

[γ-^{32}P] GTP co-migrated with non-radioactive GTP by thin layer chromatography on PEI-cellulose plastic sheets, and no difference was observed among the [γ-^{32}P] GTP supplied by Amersham, Dupont-NEN Research Products, and ICN Biomedicals (Data not shown). Thin layer chromatography on PEI-cellulose plates also showed that incubation with alkaline phosphatase completely hydrolyzed the radioactive nucleotide to [^{32}P] P_i. (Data not shown).

The integrity of the [γ-^{32}P] GTP employed in the GTPase assay was analyzed by PEI-cellulose thin layer chromatography, using 1 M LiCl as the solvent. To quantitate the amount of free, radiolabeled, inorganic phosphate ([^{32}P] P_i) present in the [γ-^{32}P] GTP obtained commercially, an aliquot of the radioactive nucleotide (300 pmol) was mixed in the assay buffer solution [20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA] alone, with rhodopsin (5 pmol), or with transducin (300 pmol). The reactions were performed in a final volume of 100 µL. An aliquot (20 µL) of these three samples was removed and added to 200 µL of a molybdic acid solution (10.1 mM molybdic acid, 1.3 M H_2SO_4). Then, an aliquot (100 µL) of a reducing solution (230 mM $Na_2S_2O_5$, 15.9 mM Na_2SO_3, and 4.2 mM 1-amino-2-naphthol-4-sulfonic acid) was also added and mixed vigorously. Finally, the mixture was extracted by vortexing with 700 µL of isoamyl alcohol. After phase separation by centrifugation, 600 µL of the organic layer was analyzed for [^{32}P] P_i by scintillation counting in 8 mL of Optiphase Hisafe II. In all cases, the amount of P_i present in the initial [γ-^{32}P] GTP samples was only 1.5-2.5% of the total, suggesting that the nucleotide was 97.5-98.5% pure. Furthermore, neither rhodopsin nor

transducin was able to hydrolyze the GTP, independently.

GTPase assay.

Transducin GTP hydrolysis assays were carried out in 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM EDTA, at room temperature (21-23°C). The amount of transducin used in each individual assay was 300 pmol. The reaction mixtures were prepared without rhodopsin and [γ-^{32}P] GTP, and rhodopsin (2.5, 5, or 10 pmol) was added immediately in the dark. Rhodopsin employed in the assays was purified by immunoaffinity chromatography under the four treatments previously described (samples #1-#4). One minute after shining light on the assay tube, using a slide projector with a 450-nm cut off filter, the reaction was started by the addition of [γ-^{32}P] GTP (600 or 1,200 pmol). Transducin GTPase activity was then followed kinetically as described in detail elsewhere (Bubis, 1995).

Effect of detergents and lipids on the light-dependent GTPase activity of transducin.

Rhodopsin samples (samples #1-#4) were combined with various concentrations (0-0.15%) of PC to measure the photodependent stimulation of transducin GTP hydrolytic activity. The commercial PC, which was dissolved in $CHCl_3$:CH_3OH, was dried down under a stream of argon, and then *in vacuo*. The lipid was re-dissolved in 1.5% CHAPS, 20 mM Tris-HCl (pH 7.2), and 100 mM NaCl, to obtain a final concentration of 15 mg PC/mL. After mixing the assay buffer with the solution of PC, the purified rhodopsin samples (2.5, 5, or 10 pmol) were added to the mixtures, in the dark. After a 5 min incubation at room temperature, transducin (300 pmol) was also added and the samples were illuminated for 1 min. Then, the reaction was started by the addition of [γ-^{32}P] GTP (600 or 1,200 pmol). Transducin GTPase activity was determined measuring the amount of [^{32}P] P_i released as a function of time, as described above. The final concentration of detergent in the assay was either 0.02% DM for the DM-solubilized rhodopsin, or 0.1% digitonin for the digitonin-solubilized rhodopsin. As PC was re-dissolved in a buffer containing CHAPS, the final concentration of CHAPS in the assay varied from 0-0.1%, depending on the amount of lipid added.

Other procedures.

Protein concentration was measured as reported by Bradford (1976). Electrophoresis on polyacrylamide slab gels (10%, 1.5 mm thick) were performed in the presence of SDS according to Laemmli (1970).

RESULTS

Purification of rhodopsin by immunoaffinity chromatography.

Table I presents a summary of the various steps employed for the purification of the four samples of rhodopsin. As can be seen in Figure 1, the four procedures rendered rhodopsin with its characteristic absorption spectrum. Rhodopsin purity is frequently measured by determining the ratio of the absorbance at 280 nm to the absorbance at 500 nm. Purified rhodopsin typically has the spectral ratio $A_{280\ nm}/A_{500\ nm}$ of 1.7-1.8 (Hong *et al*, 1982; Litman, 1982). As can be seen in Table I, the spectral ratio $A_{280\ nm}/A_{500\ nm}$ obtained for the four samples ranged from 1.76-1.9, suggesting that the isolated photopigment was very pure in all cases, with no indication of bleaching and/or contamination by some other proteins than rhodopsin. Table I also shows the concentration of rhodopsin obtained in each case.

When an aliquot of each rhodopsin sample (5 µg) was analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining, a unique polypeptide band migrating at an apparent molecular weight of 38,000 was detected (Data not shown).

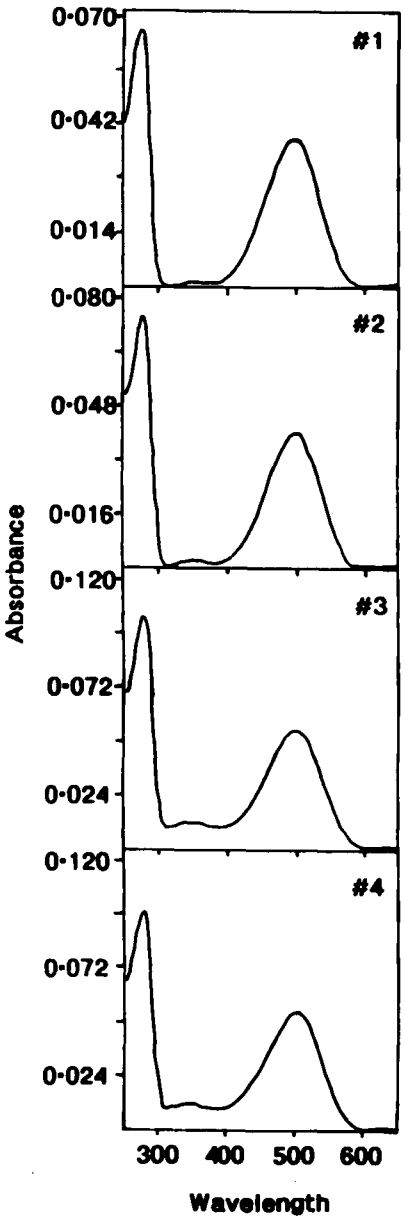

Fig 1. Absorption spectra of immunopurified rhodopsin samples. Absorption spectra of photoreceptor protein isolated following the four treatments summarized in Table I (#1-#4 correspond to rhodopsin samples #1-#4, respectively).

Transducin photoactivation by detergent-solubilized rhodopsin.

Control experiments that were performed with transducin and various concentrations of 1'-18' synthetic peptide, in the absence of rhodopsin, showed that the peptide did not stimulate the GTPase activity of transducin in the light or in the dark (Data not shown). The four samples of rhodopsin that were isolated by immunoaffinity chromatography as described above, were tested for their ability to stimulate the light-dependent GTPase activity of transducin. Figure 2 shows that rhodopsin eluted from the column with buffer containing digitonin, following the solubilization of ROS with CHAPS (Sample #1), did not stimulate the photodependent GTP hydrolytic activity of transducin. Rhodopsin that was solubilized and eluted in the presence of digitonin (Sample #2), stimulated very poorly the GTPase activity of the protein (1 pmol of GTP hydrolyzed/min/pmol of rhodopsin). On the other hand, the rhodopsin samples in which the ROS membranes were solubilized with a solution containing either CHAPS or DM, and the protein was subsequently purified in the presence of DM (samples #3 and #4), activated the GTPase capacity of transducin to 5 pmol of P_i released/min/pmol of rhodopsin. As

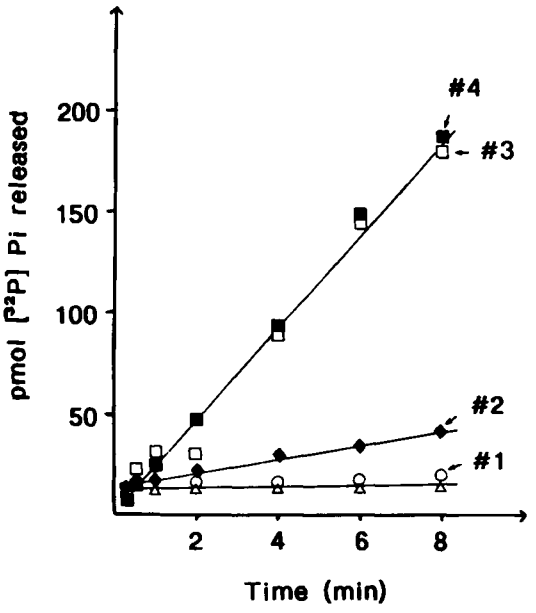

Fig 2. Stimulation of transducin GTPase activity by rhodopsin solubilized with different detergents. Rhodopsin (5 pmol) purified as described in Table I was used to induce light-dependent GTPase activity of transducin. #1-#4 correspond to rhodopsin samples #1-#4, respectively. An assay containing transducin alone was performed as control (open triangles).

expected, the GTP hydrolytic activity of transducin was undetectable in the absence of photopigment (Fig 2). When the concentration of rhodopsin was duplicated in parallel assays containing samples #1 and #2, a similar degree of stimulation on the light-dependent GTPase activity of transducin was achieved (Fig 3). These results suggest that the amount of rhodopsin in the assay mixture is not the limiting factor. Control experiments performed in the dark using the same rhodopsin samples are also shown in Figure 3.

Effect of lipids on the photodependent GTPase activity of transducin.

Addition of PC to the four different rhodopsin samples produced a substantial increase in the light-dependent GTP hydrolytic activity of transducin. As illustrated in Figure 4, for a fixed concentration of 0.075% PC, the initial rate

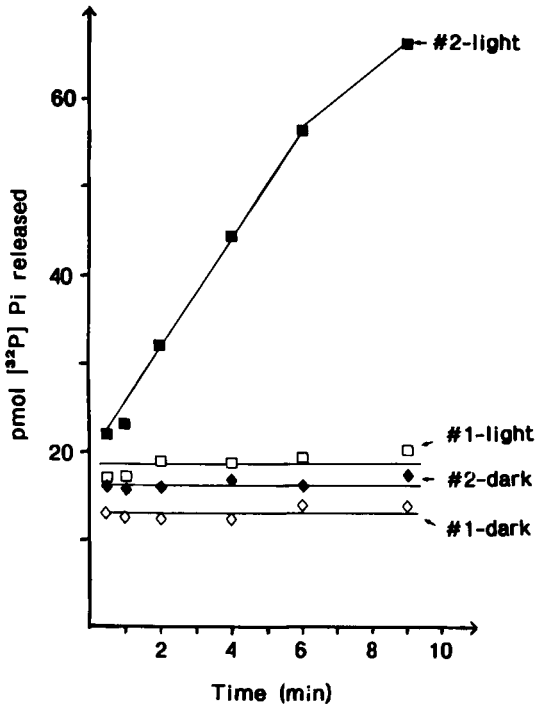

Fig 3. Stimulation of transducin GTPase activity by rhodopsin purified in presence of 1% digitonin, following ROS solubilization with buffer containing either 1% CHAPS or 1% digitonin. Rhodopsin sample #1 or 2 (10 pmol) used to induce light-dependent GTP hydrolytic activity of transducin. Assays were performed in the dark as control experiments.

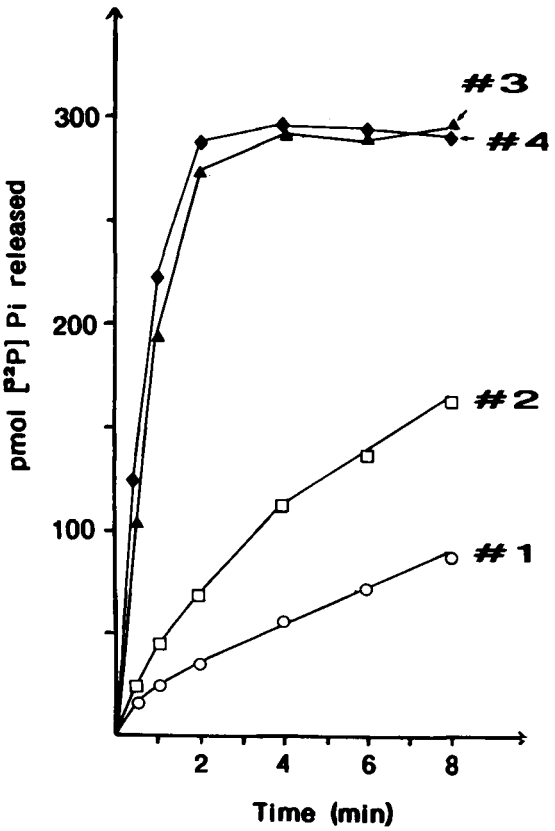

Fig 4. Effect of addition of PC on photodependent GTPase activity of transducin stimulated by different detergent-solubilized rhodopsin samples. Rhodopsin samples (#1 and #2 = 10 pmol; #3 and #4 = 5 pmol) used to induce light-dependent GTP hydrolytic activity of transducin in presence of 0.075% PC.

of light-stimulated GTP hydrolysis by transducin increased to 1 pmol of P_i released/min/pmol of rhodopsin for Sample #1, to 2 pmol of P_i released/min/pmol of rhodopsin for Sample #2, and to 30 pmol of P_i released/min/pmol of rhodopsin for samples #3 and #4.

To evaluate the lipid effect on the photodependent stimulation of transducin GTPase activity by the four rhodopsin samples, increasing concentrations (0-0.15%) of PC were added to the assays. As illustrated in Figure 5, after a 4 min incubation, rhodopsin samples #3 and #4 (2.5 pmol) induced hydrolysis of the maximum amount of nucleotide by transducin (≈ 280 pmol of GTP) when a very low concentration of PC (0.0075%) was utilized. A plateau was then reached as the concentration of lipid increased (Fig 5).

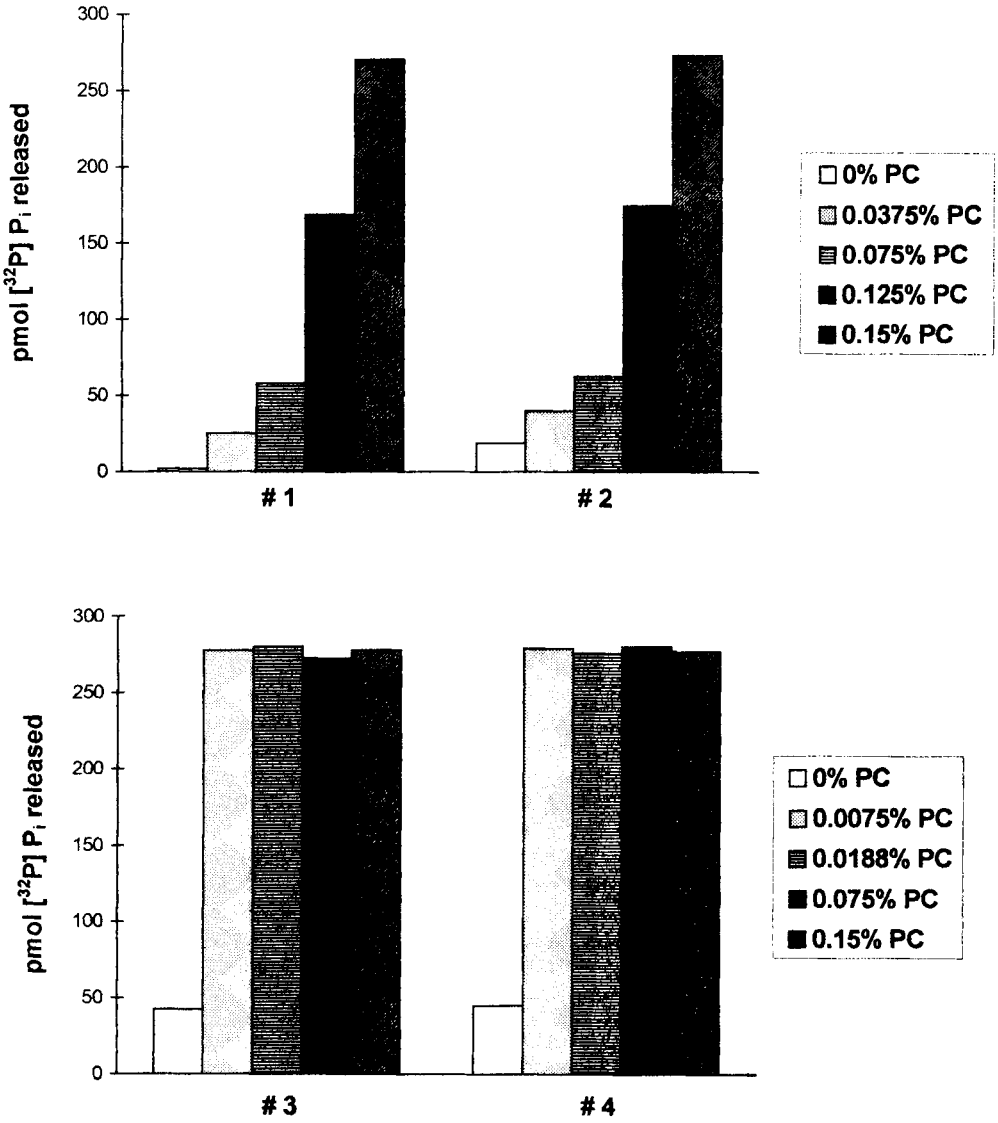

Fig 5. Effect of increasing the concentration of PC on light-dependent GTP hydrolytic activity of transducin stimulated by different detergent-solubilized rhodopsin samples. Rhodopsin samples [#1 and #2 = 10 pmol (top panel), #3 and #4 = 2.5 pmol (bottom panel)] were used to induce light-dependent GTPase activity of transducin in presence of various concentrations of PC. Amount of P_i released after 4-min incubation. Insets in both panels, concentrations of PC used in experiments.

However, rhodopsin samples #1 and #2 only stimulated the GTPase activity of transducin to the same maximum value in the presence of 0.15% PC, and when the amount of rhodopsin was increased 4-fold in the assay mixture (10 pmol) (Fig 5). Furthermore, as also illustrated in Figure 5 for the 4-min period, the rate of GTP hydrolysis increased as a function of the lipid concentration when samples #1 and #2 were used to stimulate the GTPase activity of transducin (data not shown). Control experiments that were performed in the dark with the four rhodopsin samples and in the presence of various concentrations of PC (0-0.15%) showed no GTP hydrolytic activity of transducin (Data not shown).

DISCUSSION

A common feature of all photobiological systems is the tight association of the photopigments with highly organized

membrane structures. This arrangement is typified by the disks of the retinal ROS. The visual pigment rhodopsin constitutes about 90% of the disk membrane protein and is thought to have the major portion of its mass embedded within the phospholipid bilayer of the disk membrane (Saibil *et al*, 1976). Low resolution structural models of rhodopsin have been derived from two-dimensional crystals (Schertler *et al*, 1993; Unger & Schertler, 1995; Schertler & Hargrave, 1995), and the results are consistent with the assumption that the disk membrane is composed largely of rhodopsin imbedded within a lipid matrix. As such, one might anticipate that there will be an intimate relationship between visual pigment function and the microenvironment provided by the lipid matrix of the disk membrane.

Although the stability of rhodopsin in detergent solutions has long been a matter of concern (DeGrip, 1982; and references therein), the manner in which surfactants affect the lipid(s) concomitantly extracted with rhodopsin under comparable solubilization conditions, has yet to be explored. In this work, different detergents were used to investigate the light-dependent stimulation of transducin by solubilized rhodopsin. Rhodopsin samples eluted from the column with DM, following ROS solubilization with buffer containing either CHAPS or DM, retained the ability to stimulate the GTPase capacity of transducin. However, the interaction with transducin was impaired when rhodopsin purified in the presence of digitonin, following ROS solubilization with digitonin, was employed in the assay. A complete loss of light-stimulation on the transducin GTPase activity was observed with rhodopsin samples that were initially treated with buffer containing CHAPS for the solubilization of the ROS membranes, and then eluted from the column in the presence of digitonin. Thus, the combination of CHAPS and digitonin during the purification steps of rhodopsin appeared to have a synergistic negative effect on the stimulation of the photodependent GTPase activity of transducin by the solubilized pigment. In all the cases examined, addition of PC to the solubilized rhodopsin samples increased the light stimulated GTP hydrolysis catalyzed by transducin. These results suggest that the effective light-stimulation of transducin by the photoreceptor protein requires phospholipids, which appeared to be differentially eliminated with the detergents used for the solubilization of ROS and the purification of rhodopsin.

Glycerophospholipids [phosphatidylcholine (41%), phosphatidyl ethanolamine (39%), and phosphatidylserine (13%)] constitute more than 90% of the total lipid present in disks isolated from bovine retina (Anderson & Maude, 1970; Daemen, 1973). The behavior of native disk phospholipids in the process of rhodopsin solubilization was investigated by Aveldaño (1995) using five different detergents, two nonpolar amphiphiles - Emulphogene (polyoxyethylene-10 tridecylether), and octylglucoside (N-octyl-β-D-glucopyranoside)- and three which have a rigid alicyclic ring system - taurocholate, CHAPS, and digitonin-. Aveldaño (1995) has shown that these detergents differ dramatically in their ability to extract lipid and rhodopsin, both on an absolute and on a relative basis. Aveldaño also shows that there is a certain selectivity in the distribution of phospholipid classes in the aqueous phase between rhodopsin-lipid-detergent and lipid-detergent micelles, during the solubilization process (Aveldaño, 1995). Thus, the nature of the lipid(s) bound to the solubilized rhodopsin appears to vary depending on the surfactant employed. The results presented here suggest that the purification of the protein in the presence of DM, after solubilization of ROS membranes with buffer containing either CHAPS or DM, allows the association to rhodopsin of certain amount of the type(s) of phospholipids which is (are) required for the light-dependent stimulation of transducin. On the other hand, the purification of rhodopsin in the presence of digitonin, following solubilization of ROS membranes with digitonin, seems to eliminate the majority of these phospholipids. When CHAPS was employed during the solubilization of the ROS, prior to rhodopsin purification in the

presence of digitonin, these phospholipids were completely eliminated. The absence of these phospholipids associated with rhodopsin, in turn hinders its productive interaction with transducin.

The detergents used for the solubilization of ROS membranes and during the elution of rhodopsin from the column, as well as the presence or absence of lipids, also may influence the stability of the reactive rhodopsin photointermediates produced upon illumination. Applebury *et al* (1974) have shown a dramatic increase in the kinetics of the Meta I to Meta II transition (two orders of magnitude faster) for N,N-dimethyldodecylamine oxide-solubilized, phospholipid-free rhodopsin, relative to rhodopsin bound to ROS membranes. Litman *et al* (1981) utilizing octylglucoside-solubilized rhodopsin samples containing varying amounts of associated disk phospholipid, also reported that the rate constant for the formation of Meta II increased (≈ 3-fold) as the molar ratio of phospholipid per rhodopsin decreased from 35 to 5. O'Brien *et al* (1977) examined the photochemical functionality of purified rhodopsin incorporated into phospholipid bilayers. They found that recombinant membranes prepared from unsaturated phospholipids, *e.g.*, asolectin, egg phosphatidylcholine, egg phosphatidyl ethanolamine, and dioleoyphosphatidylcholine, showed first-order kinetics for the transition with rates comparable to that of ROS (O'Brien *et al*, 1977). On the other hand, recombinants prepared from saturated phosphatidylcholines, *e.g.*, dipalmitoylphosphatidylcholine, had a retarded rate of conversion from Meta I to Meta II and were considered to be non-functional (O'Brien *et al*, 1977). Baldwin and Hubbell (1985a) also have shown that photolysis of rhodopsin in dimyristoylphosphatidylcholine (a saturated short- chain phospholipid) recombinant membranes resulted in an abnormal sequence of spectral transitions, and the dominant product of Meta I decay was free retinal plus opsin. In an accompanying paper, Baldwin and Hubbell (1985b) determined the properties of the lipid environment that were responsible for such altered spectral behavior, by incorporating rhodopsin into a series of phosphatidylcholines of defined composition, and found that Meta II was not formed in appreciable amounts in bilayers containing acyl chains that are too short (14 or fewer carbon atoms in length), in the presence of only n-alkyl chains, or below the characteristic phase-transition temperature of recombinant membranes. They also determined that double bonds were not required for the formation of the Meta II intermediate (Baldwin & Hubbell, 1985b). Finally, Aveldaño (1995) has shown that rhodopsin photolytic transitions were faster in nonionic than in bile salt-related detergent. On this basis, it is plausible that the production of light activated rhodopsin or Meta II, is slower in digitonin than in DM, and maybe an earlier rhodopsin photointermediate is actually being trapped upon illumination when digitonin is present in the rhodopsin sample. Furthermore, the permanence of phospholipids associated to rhodopsin depending on the detergent utilized during ROS solubilization and photoreceptor protein isolation, may also influence the rate of Meta I to Meta II transition, and consequently the light-dependent stimulation of transducin.

Even though most of the previous discussion has been focused on the effect of the detergents and lipids on rhodopsin, one could also speculate on the possibility that they might be affecting directly the structure and/or function of transducin too. As described in the text, transducin is a peripheral enzyme, isolated from the ROS membranes, and it is purified in a soluble form by standard column chromatography, without the use of detergents (see Methods). On the other hand, rhodopsin is an integral membrane protein, which is tightly associated with the interior of the ROS membranes, and requires the use of high concentrations of detergents for its solubilization and purification (Table I). The detergents included in the four different extraction protocols used for the isolation of rhodopsin are non-denaturing reagents. Furthermore, the detergent concentration in the GTPase activity assay

is lowered to one tenth of the original concentration present in the rhodopsin samples. Under these conditions, the conformation and function of a soluble protein like transducin are expected to remain unchanged. Since transducin interacts peripherally with the ROS membranes, its structure and biochemical behavior is probably not affected either by the presence of PC. Based on these assumptions, although it is not possible to discard a direct influence of the detergents and lipids on transducin, it is more likely that they are playing a direct role on the structure and/or function of the photopigment rhodopsin.

The data presented in this work emphasize the point that care should be exercised in extrapolating results obtained with detergent-solubilized membrane proteins to the native membrane situation. Although different detergents exhibit similar properties, this does not necessarily make them fully interchangeable. Bearing this in mind may provide an explanation for the apparently erratic behavior of some membrane proteins after solubilization and lead to improvements in experimental protocols that involve detergents. Further studies will be necessary to establish the effect of other detergents used for the solubilization of ROS membranes and isolation of rhodopsin. The addition of highly unsaturated lipids (soybean lipids, brain phosphatidylethanolamine/ phosphatidylcholine, *etc*) on the production of Meta II and subsequent transducin activation also should be explored. Finally, the effects of different detergent-phospholipid mixtures on other light-dependent reactions of rhodopsin, like the photodependent phosphorylation of rhodopsin by rhodopsin kinase and arrestin binding to the phosphorylated protein, might also be affected.

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