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Cyclic nucleotides and GTP analogues stimulate light-induced phosphorylation of octopus rhodopsin

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Light-induced phosphorylation of octopus rhodopsin in microvillar membrane was shown to be stimulated by cyclic nucleotides in contrast to vertebrate rhodopsin kinase. Non-hydrolyzable GTP analogues, GTPγS and GppNHp, greatly enhanced the light-induced phosphorylation of octopus rhodopsin, but the non-hydrolyzable GDP analogue, GDPβS, was not effective. These results suggest that rhodopsin A-kinase is involved in regulating the interaction between rhodopsin and G-protein in octopus photoreceptors.

Rhodopsin; Rhodopsin kinase; Kinase, A-; Protein, G-; Photoreceptor; (Octopus, Invertebrate)

1. INTRODUCTION

For the phototransduction in vertebrate photoreceptors it has now been established that G-protein (transducin) couples the photoexcited rhodopsin to cGMP hydrolysis and cGMP controls the cation permeability in plasma membranes [1]. The recovery of light activation must begin with inactivation of the receptor. Rhodopsin is phosphorylated by an intrinsic kinase after exposure to light [2,3]. Although the physiological significance of rhodopsin phosphorylation is not yet understood, it is thought to be associated with desensitization of rhodopsin coupled to cGMP metabolism [4].

Homologous receptor desensitization was observed when the ligand-bound form of the α_1- and β_2-adrenergic receptors was phosphorylated [5,6]. Though phosphorylation of the α_2-adrenergic receptor is enhanced by cAMP [6], cyclic nucleotides have no direct role in regulating rhodopsin kinase activity in vertebrate photoreceptors [7].

In the present work, we report that the invertebrate rhodopsin kinase was enhanced by cyclic nucleotides as in the adrenergic receptor kinase. Moreover, we studied the effect of guanylate nucleotides on phosphorylation of rhodopsin and found that activation of G-protein by GTPγS strongly enhanced phosphorylation of illuminated octopus rhodopsin, suggesting that rhodopsin kinase is involved in regulating the interaction between rhodopsin and G-protein in invertebrate photoreceptors.

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2. MATERIALS AND METHODS

2.1. *Preparation of octopus photoreceptors*

Octopus photoreceptor membranes were prepared essentially the same as previously described [8], with minor modifications as follows: retinas from thawed octopus eyes were suspended in the modified cephalopod saline, buffer A (400 mM KCl, 5 mM $MgCl_2$, 5 mM MOPS pH 7.4, 1 mM dithiothreitol (DTT), 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (P-APMSF), 5 μg/ml leupeptin, 5 μg/ml trypsin inhibitor, 1 μg/ml pepstatin), and were spun down at 18 000 rpm in a refrigerated centrifuge for 20 min. The pellet was mixed with 34% sucrose (w/v) in buffer A (sucrose solution) and centrifuged at 18 000 rpm for 30 min. The supernatant was diluted with an equal volume of buffer A and centrifuged. The pellet was resuspended in the fresh sucrose solution and the above procedure was repeated. The resultant pellet containing microvillar membranes were then washed 4 times with buffer A followed by washing 4 times with buffer B (5 mM Tris-Cl, pH 7.4; 1 mM DTT, 10 μM P-APMSF, 5 μg/ml leupeptin, 5 μg/ml trypsin inhibitor and 1 μg/ml pepstatin). The fluffy fraction of the pellet was collected and used for assay.

2.2. *Assay for rhodopsin kinase*

Phosphorylation of rhodopsin was carried out at 20°C in a final volume of 20 μl containing 50 mM photassium phosphate pH 7.2, 100 mM KCl, 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (5 μCi/sample), 500 μg protein in microvillar membranes. Phosphorylation was initiated by adding the [γ-^{32}P]ATP in the dark. For studies of light-induced phosphorylation, samples were irradiated immediately after the addition of the [γ-^{32}P]ATP in the dark. The reaction was terminated by dilution with 20 μl of electrophoresis buffer containing 2% SDS, 200 mM Tris pH 8.6, 2% mercaptoethanol, 20% glycerol and 0.01% bromophenol blue. Aliquots (10 μl) of radiolabeled membranes dissolved in the electrophoresis buffer were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) on slab gel (1 mm thick) containing 12% acrylamide in the running gel

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and 4% acrylamide in the stacking gel. ^{32}P-labeling of proteins was detected by autoradiography (Kodak X-Omat film). For quantitative determination, rhodopsin bands at M_r 50 000 were cut out from slab gels and incorporation of ^{32}P was estimated by scintillation counting.

3. RESULTS AND DISCUSSION

The autoradiogram in fig.1B (lane b) illustrates that, in the dark, phosphate was incorporated primarily into a low-molecular-weight region, which migrated with tracking dye and was characterized as inositol phospholipids [9]. Illumination of washed microvillar membranes induced a marked increase in broad bands centered at M_r 50 000 and at M_r 94 000, identified as rhodopsin and its dimer, respectively. Time course of phosphorylation of rhodopsin was studied in the dark and in the light as shown in fig.1A. In the dark, the phosphorylation without cyclic nucleotide reached plateau in about 10 min. The addition of cGMP (10 μM) increased dark phosphorylation more than twice, while cAMP (10 μM) failed to enhance the reaction. The phosphorylation with cyclic nucleotides reached maximum at 15 min after onset of illumination and declined thereafter. Light-induced phosphorylation was stimulated by cAMP slightly more than by cGMP. Thus, the stimulatory effect of cAMP was more prominent than that of cGMP. Dephosphorylation of the labeled phosphate may be induced by intrinsic phosphatase in the microvillar membranes.

The effects of various concentrations of cAMP and cGMP on phosphorylation of octopus rhodopsin in illuminated microvillar membranes are shown in fig.2. Both cAMP and cGMP enhanced phosphorylation of illuminated octopus rhodopsin, and cAMP was more effective. The apparent K_m for cAMP was about 0.03 μM and that for cGMP was about 1 μM, which indicates that cAMP was about 30 times more effective than cGMP in activating octopus rhodopsin kinase. These results suggest that the illuminated octopus rhodopsin was phosphorylated by a cAMP-dependent kinase (an A-kinase) as with β_2 adrenergic receptor kinase [5]. However, stimulation of phosphorylation of octopus rhodopsin by cAMP was at most 30%, which is less than the other cyclic nucleotide-dependent kinase. It is possible that another rhodopsin kinase which is independent of cyclic nucleotide might be functional in octopus photoreceptor. Another explanation is that endogeneous cyclic nucleotide was strongly bound to the kinase, hence the activation by externally added cAMP was only partial.

In vertebrate photoreceptors, the effect of cyclic nucleotide on rhodopsin kinase has been subject to discussion [10,11]. Recently, Palczewski et al. [7] reinvestigated the effects of cyclic nucleotides using purified bovine rhodopsin kinase and urea-washed ROS membranes. They found only a small effect of these compounds on vertebrate rhodopsin kinase and concluded that cyclic nucleotide had no direct role in regulating rhodopsin kinase activity in vertebrate photoreceptors. The effect of cyclic nucleotides on phosphorylation of invertebrate rhodopsin was examined by Paulsen and Hoppe [12]. They showed that neither cGMP nor cAMP at 0.5 mM stimulated phosphorylation of octopus rhodopsin in the presence or absence of light. However, present data demonstrated that both cAMP and cGMP enhanced phosphorylation of illuminated rhodopsin in octopus photoreceptor membranes. As shown in fig.2, stimulation of phosphorylation decreased at a higher concentration of cAMP and cGMP. This may be the reason why Paulsen

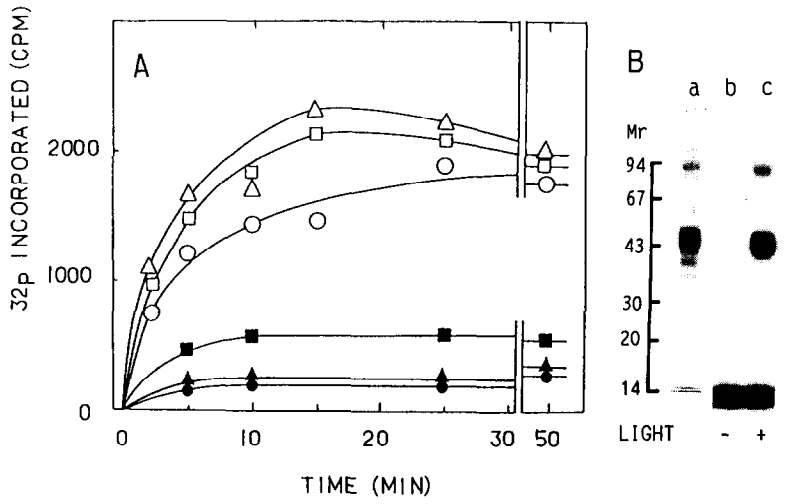

Fig.1. (A) Time course of light-induced phosphorylation of octopus rhodopsin in the presence and absence of cyclic nucleotide. Washed microvillar membranes in buffer A were preincubated without (○,●) or with 10 M cAMP ((△,▲) and 10 μM cGMP (□,■) for 10 min in the dark. Buffer A containing 100 μM [γ-^{32}P]ATP (5 μCi) was mixed with the membrane solution just before onset of light. The membrane solutions were then continuously in the light ((○,△,□) or in the dark (●,▲,■). (B) Phosphorylation of octopus rhodopsin. Coomassie blue stained gel of washed microvillar membranes (a). Autoradiogram of phosphorylation of octopus microvillar membranes in the dark (b) and in the light (c).

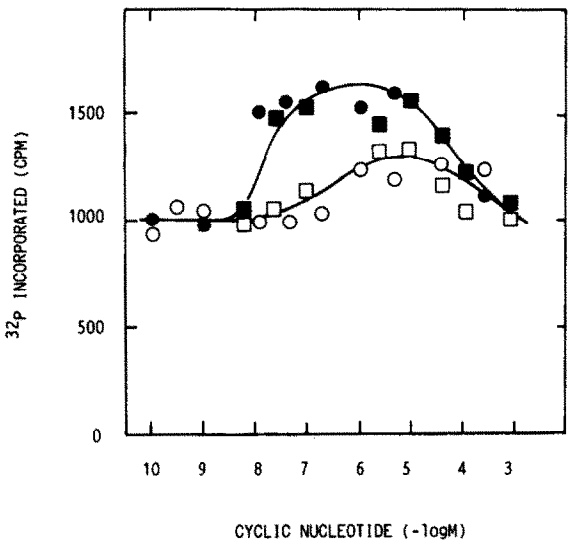

Fig.2. Phosphorylation of illuminated octopus rhodopsin in the washed microvillar membranes in the presence of varying concentrations of cAMP (■,●) and cGMP (□,○). Assay conditions were as described in section 2, except for the variation in the cyclic nucleotide concentration.

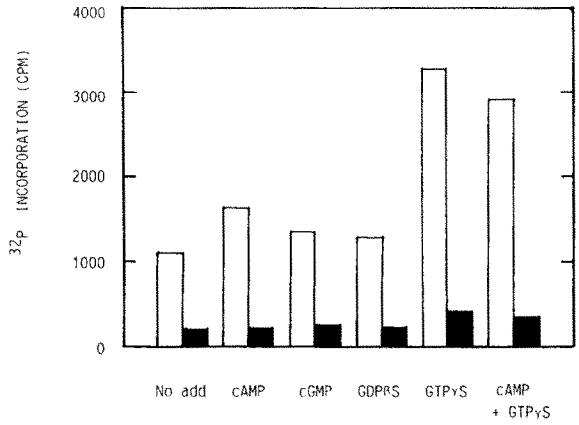

Fig.3. Effect of nucleotides on light-induced phosphorylation of octopus rhodopsin. Washed microvillar membranes were preincubated with buffer A containing nucleotides as indicated for 10 min. The phosphorylation reaction took place for 20 min in the presence (□) or absence of light (■).

and Hoppe failed to observe stimulation by cAMP and cGMP at 0.5 mM. Though cAMP did not affect the dark phosphorylation of octopus rhodopsin, cGMP enhanced it a little. This mechanism is open for further studies.

We next looked at the effect of guanyl nucleotides on phosphorylation of octopus rhodopsin. Washed microvillar membranes were preincubated with or without nucleotides and the phosphorylation reaction was started by adding [γ-^{32}P]ATP either in the absence or in the presence of light. Fig.3 shows quantitative value of incorporation of ^{32}P into octopus rhodopsin. As presented in fig.1, a significant amount of octopus rhodopsin was phosphorylated without nucleotides in the presence of light, and cyclic nucleotides stimulated phosphorylation of illuminated rhodopsin. On the other hand, non-hydrolyzable GTP analogs, GTPγS and GppNHp, stimulated phosphorylation of octopus rhodopsin by about two-fold, though cAMP inhibited slightly GPTγS stimulated phosphorylation of octopus rhodopsin. In contrast, non-hydrolyzable GDP analogue, GDPβS, failed to stimulate the reaction. These results indicated possible involvement of G-protein in phosphorylation of illuminated octopus rhodopsin.

It is known that GTPγS can retain G-protein in an active state and α-subunit of G-protein is released from receptor [1]. Thus, the dissociation of α-subunit of G-protein from octopus rhodopsin would uncover another phosphorylation site(s) for rhodopsin kinase. These results suggest that phosphorylated site of octopus rhodopsin stimulated by GTP analogues may be involved in the binding surface for protein of octopus photoreceptor [13]. Our preliminary results show that possible phosphorylated sites for octopus rhodopsin enhanced by GTPγS were Ser319, Thr329, Thr330, Thr336, which suggest that the G-protein associates with C-terminal side in the seventh helix of octopus rhodopsin. In other words, phosphorylation of octopus rhodopsin at this surface might inhibit association of G-protein with rhodopsin, which might lead to the desensitization of the transduction cascade [4].

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