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CYCLIC NUCLEOTIDE PHOSPHODIESTERASES ASSOCIATED WITH BOVINE RETINAL OUTER-SEGMENT FRAGMENTS*

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Summary

ATP-dependent cyclic GMP phosphodiesterase activity (EC 3.1.4.16) associated with bovine retinal outer-segment fragment preparations was stimulated an order of magnitude by light, confirming the results of Miki et al. (1973) Proc. Natl. Acad. Sci. U.S. 70, 3820—3824 at Yale for the frog system. In contrast to the results of the Yale group, however, light stimulation was not observed for cyclic AMP as substrate. A direct relationship of bovine rhodopsin bleaching to phosphodiesterase activation differs from a previous report by the Yale group that full activation of the frog enzyme was achieved by bleaching of a maximum of 2% rhodopsin.

Phosphodiesterase activity could be qualitatively removed from the fresh outer-segment preparations with isotonic sucrose which apparently did not disrupt the plasmalemma or discs. Activity recovered from the washing was not light sensitive.

Two K_m values were determined for cyclic AMP, 5 and 0.05 mM; for cyclic GMP a K_m of 0.22 mM was found. All K_m values were determined in the presence of 1 mM ATP in the dark. Sonication of fresh outer segments or storing at −20° C abolished the light response. However, storage at −76° C fully preserved it.

Introduction

The role of cyclic nucleotides in the function of vertebrate retinal outer segments is insufficiently understood. The question of whether adenylate cyclase in the retinal segments is either light inhibited, or of exceptionally high

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activity as earlier reported by Bitensky's laboratory at Yale [1—4] has now been resolved. At least three laboratories, including our own, have reported inability to reproduce these results [5—7]. Bitensky's group has also reported reinterpretation of their original data, as well as new data which appear to indicate that the effect of light is to stimulate cyclic nucleotide phosphodiesterase, rather than to inhibit cyclase [8]. The present report confirms the existence of a light-activated, ATP-dependent cyclic GMP phosphodiesterase associated with bovine retinal outer-segment preparations.

Methods

Chemicals and reagents. Cyclic [8-^{3}H]AMP (17.5 Ci/mmol), cyclic [8-^{3}H]GMP (14.3 Ci/mmol) were purchased from International Chemical and Nuclear; ultrafiltration membranes from Amicon; sodium phosphate from Baker; hydroxylamine hydrochloride, $MgCl_2$, naphthalene, ammonium acetate from Mallinckrodt; POPOP and PPO from Research Products International; *p*-dioxane from Eastman; sucrose from Fischer. All other reagents and enzymes were purchased from Sigma, and chromatography paper (Whatman 3 MM) from Scientific Products.

Outer-segment preparations. Bovine retinal outer-segment fragments were isolated from fresh bovine retinas by the method of Mc Connell [9]. These preparations consistently exhibit $A_{280\,nm}/\Delta A_{498\,nm}$ ratios of from 2.5 to 3.5, contain very little particulate debris as determined by electron microscopy, and contain $\leqslant$1% mitochondrial contamination as determined by cytochrome oxidase assays [9]. The yield from 400 bovine retinas is typically from 100 to 150 mg Lowry protein [10] of outer segments. Various derivative fractions were prepared by methods detailed below. Unless otherwise indicated, all experiments were performed on fresh outer-segment fragments. Electron microscopy of either fresh or frozen fragments reveals largely intact plasmalemmas enclosing closely packed stacks of intact discs. In some cases fragments are rolled into doughnut shapes, a result of homogenization.

Assays. Cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase were assayed in a reaction volume of 150 μl including 30 mM Tris · HCl (the pH was 7.5), 3 mM $MgCl_2$, 0.25 M sucrose, 2 mM 8-^{3}H-labeled cyclic nucleotide (100—200 cpm/nmol) with or without 1 mM ATP and 0.1—0.25 mg retinal outer-segment protein, determined by the method of Lowry et al. [10]. Assay temperature was 30°C. Assay was initiated by addition of either retinal outer segments or cyclic nucleotide, in either case preincubated at 30°C, as was the mixture of other components, in a Dubnoff shaker. After a predetermined time in the shaker, the assay, or aliquots thereof, were terminated by boiling for 2 min. Cooling and precipitation by clinical centrifuge followed. Aliquots (50 or 100 μl) of the supernatant fluid were spotted on 23 × 57 cm Whatman 3MM chromatography paper and developed for 18—20 h with 95% ethanol/1 M ammonium acetate (73 : 27, v/v) in a descending manner. In our hands this technique separates cyclic AMP and cyclic GMP from ATP, ADP, AMP, adenosine, GTP, GDP, GMP, guanosine, XTP and hypoxanthine. The cyclic nucleotide spots were visualized by ultraviolet light, cut out, eluted with 2 ml water, placed in 10 ml Bray's solution [11] and counted in a Packard TriCarb liquid

scintillation system. Two control assays were run on boiled outer-segment aliquots for every three experimental assays, making a total of five spots per chromatography sheet. Specific activity was therefore calculated after subtracting experimental counts from control counts. In this way disappearance of cyclic nucleotide was measured directly.

Unbleached rhodopsin was assayed as previously described [7].

Results

In confirmation of the report by the Yale group for frog rod outer segments [8] we have found a cyclic GMP phosphodiesterase associated with bovine retinal outer segments which is stimulated 7—30-fold by illumination with white light, provided 1 mM ATP is also present. In Fig. 1 are presented some typical time curves illustrating the light stimulation. In one outer-segment preparation 2 mM cyclic GMP was completely hydrolyzed within 30 s after illumination began. In contrast to the Yale group's report for frog outer segments, we observed no light stimulation in bovine outer segments when the substrate was 2 mM cyclic AMP, and even the dark activity was consistently lower than that observed when cyclic GMP was the substrate. The time curves for both cyclic GMP and cyclic AMP activities were unaffected by prior storage of outer-segment fragments at —76° C in 0.88 M buffered sucrose.

Table I shows the relationship of ATP concentration to the light activation of cyclic GMP phosphodiesterase in bovine retinal outer-segment fragments. Maximal light activation occurs at 1 mM ATP and concentrations of ATP above 5 mM inhibit both the dark and light activities. These data are in

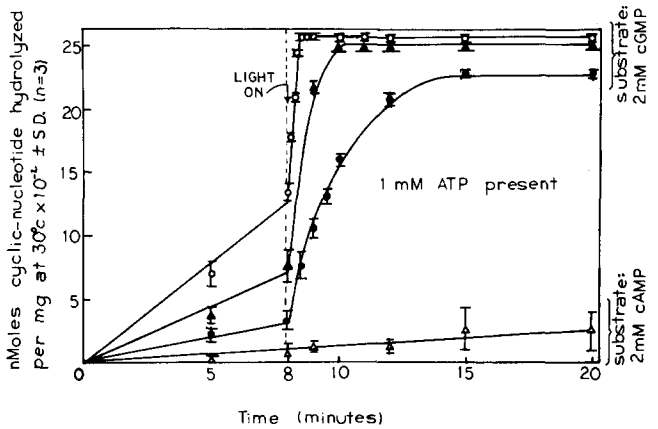


Fig. 1. Effect of illumination on cyclic nucleotide phosphodiesterase activity in three different preparations of outer-segment fragments. "Dark" incubation for first 8 min was performed under dim red light. Assays were conducted as described in Methods on frozen (—76° C) outer-segment preparations. (The same results were obtained with fresh, unfrozen preparations.) Reactions were initiated at 0 min by addition of outer-segment fragments. Reaction volume was 5 ml, and aliquots were removed and boiled at the indicated times. Illumination was provided by a 20 W, 118 V (a.c.) Westinghouse cool white fluorescent light located approx. 15 cm above reaction vessels. ○, prep. 1; ●, prep. 2; △ and ▲, prep. 3 in the presence of 2 mM cyclic AMP and cyclic GMP, respectively. Omission of ATP (a) eliminated the light effect and (b) reduced dark activity as much as 50%. The facilitating effect of ATP was observed whether the outer-segment fragments were prebleached or illuminated during assay.

TABLE I

THE COMBINED EFFECTS OF LIGHT AND ATP ON CYCLIC GMP PHOSPHODIESTERASE ACTIVITY ASSOCIATED WITH BOVINE PHOTORECEPTOR OUTER-SEGMENT FRAGMENTS

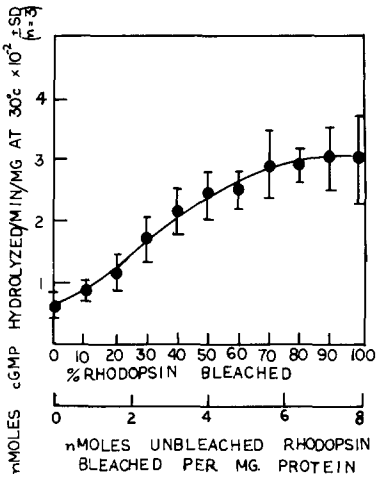
ATP (mM)	Cyclic GMP phosphodiesterase activity				Light/dark ratio
	Light		Dark		
	nmol/mg per min	Percent	nmol/mg per min	Percent	
0.00	40	100	40	100	1.0
0.10	224	560	110	275	2.0
0.50	420	1050	80	200	5.3
1.00	600	1500	80	200	7.5
2.00	240	600	60	150	4.0
5.00	40	100	40	100	1.0
10.00	10	25	10	25	1.0

good agreement with the results for frog outer segments reported by Miki et al. [8] who obtained an optimal ATP concentration of 0.75 mM. These investigators reported greatest light activation at sub-saturating (15 μM) substrate concentration [8] (Fig. 3A of ref. 8). In some contrast, Chader et al. [12,13] also studying bovine outer segments and using sub-saturating substrate concentrations reported severe inhibition of cyclic nucleotide phosphodiesterase activities at 1 mM ATP levels (Table IV of ref. 12) but showed an apparent 1.6—1.9-fold light activation of phosphodiesterase in the presence of 0.1 mM ATP concentration [12,13]. The discrepancy between optimal ATP concentrations as determined by Chader et al. [12] on the one hand and by Miki et al. [8] and our laboratory on the other hand may be due either to differences in substrate levels or to the use by Chader et al. [12] of a coupling enzyme, snake venom 5'-nucleotidase, to convert the 5'-nucleotide product of cyclic nucleotide phosphodiesterase activity to adenosine or guanosine. Snake venom 5'-nucleotidase activity is almost completely inhibited by 1 mM ATP but is not affected by 0.1 mM ATP [14]. Therefore, at higher ATP levels very little 5'-nucleotide is hydrolyzed by the nucleotidase to the nucleoside. This would be reflected by an apparent inhibition of phosphodiesterase activity, since it is only the nucleoside which is assayed when the coupling enzyme is used. Our laboratory and the Yale laboratory directly assayed unhydrolyzed cyclic nucleotide and thus did not have this problem.

Kinetic studies performed in dim red light in presence of 1 mM ATP on cyclic nucleotide phosphodiesterase activities associated with our bovine outer-segment preparations revealed two K_m values for cyclic AMP of 5 and 0.05 mM and one K_m value for cyclic GMP of 0.22 mM. These values agree well with those of Miki et al. [8] for frog outer segments. This is the only other group to report K_m values in the presence of ATP.

Bleaching, homogenization, freezing, dim red light and sonication

The Yale group reported that (a) if 2% of frog rhodopsin is bleached the cyclic nucleotide phosphodiesterase is fully activated [8,15]; (b) dim red light also fully activates the enzyme [8]; (c) homogenization (glass on glass) of



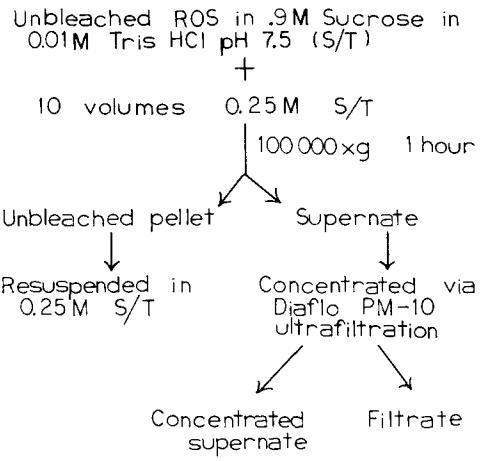


Fig. 2. Cyclic GGMP phosphodiesterase activity as a function of the amount of rhodopsin experimentally bleached. Two aliquots from the same retinal outer-segment preparation were taken; one was bleached and added in the appropriate proportion to the unbleached aliquot to make the given percentages. Rhodopsin spectra were performed as described elsewhere [7]. Assays were run for 6 min under dim red illumination. Maximum percent substrate utilized was 70% for the 100% bleached point.

Fig. 3. Flow chart for isotonic washing of freshly isolated outer-segment fragments. All operations were performed on unbleached outer-segment fragments in dim red light at 4°C, except for the ultrafiltration step at the end. There were no detectable rhodopsin or other visible pigments in the supernatant either before or after that step.

retinal outer segments in the dark fully activates the phosphodiesterase but lysis in hypotonic media does not alter the light effect [8]. Since we routinely prepare our outer segments in dim red light and use homogenization (Teflon on glass) during outer-segment isolation [9] one might predict that any light activation of cyclic nucleotide phosphodiesterase associated with our bovine preparations would be lost during the isolation procedure. However, in contrast to the Yale group we not only observe the light-activated cyclic GMP phosphodiesterase but the activation, in the presence of 1 mM ATP, is proportional to the amount of unbleached rhodopsin experimentally bleached (Fig. 2). Since the cattle from which we obtain our fresh retinas are not dark adapted prior to slaughter we estimate, based on the regeneration experiments of De Grip et al. [16] that as much as 20—50% of the rhodopsin in our preparations is prebleached prior to assay. Nevertheless, the phosphodiesterase activity is stimulated many-fold by bleaching the remaining unbleached rhodopsin. We cannot explain the apparent discrepancy between results with our bovine enzyme and the results reported for the frog enzyme by Miki et al. [8].

Either sonication or storing of bovine outer-segment fragments at −20°C will abolish the light activation and significantly diminish dark activity. However, the phosphodiesterase remains light reponsive indefinitely if preparations are stored at −76°C or below in the presence of 0.88 M sucrose.

Isotonic washing of the retinal outer-segment fragments

A flow chart describing the procedures for washing freshly isolated outer-segment fragments in 0.25 M sucrose appears in Fig. 3. Cyclic nucleotide phos-

TABLE II

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY IN FRACTIONS ASSAYED WITH AND WITHOUT WASHING IN ISOTONIC SUCROSE (SEE FIG. 3 FLOW CHART)

Assays were carried out as in caption to Fig. 1. Fractions were stored at −76°C after treatment until assay.

Fraction	Total mg protein	Cyclic GMP phosphodiesterase			
		Specific activity (nmol/mg per min)		Total activity (nmol/min)	
		Dark	Light	Dark	Light
Unwashed outer-segment fragments	76	37	465	2812	35430
Washed pellet	66	0	32	0	2112
Concentrated supernatant	4	420	420	1680	1680*
Filtrate	3	0	0	0	0
		Cyclic AMP phosphodiesterase**			
		Specific activity (nmol/mg per min)		Total activity (nmol/min)	
Unwashed outer-segment fragments	179	19.2 ± 2.3		3437	
Washed pellet	144	3.4 ± 1.5		440	
Concentrated supernatant	14	252 ± 67		3528	
Filtrate	<1				

* Cyclic GMP phosphodiesterase was washed off unbleached outer-segment fragments.

** Dark values only; there was no effect of light on cyclic AMP phosphodiesterase activity (Fig. 1). Activities were assayed by the method of Rutten et al. [19] involving 5′-nucleotidase and Dowex chromatographic separation of [^{3}H]adenosine. In these experiments, the method gives results comparable to those obtained with the method described in Methods for direct assay of unhydrolyzed cyclic nucleotide.

phodiesterase activity was assayed on unwashed and washed outer segments; on the reconcentrated supernatant fluid from the wash and a combination of washed outer-segment fragments plus concentrated supernatant. The assays were conducted on these fractions following their storage at −76° C for experimental convenience. The data presented in Table II make it clear that only the unwashed fragments retained light sensitive phosphodiesterase activity. Washing abolished light sensitivity in the other fractions. The data in Table II also disclose that while cyclic AMP phosphodiesterase activity was fully recovered in the reconcentrated supernatant, cyclic GMP activity was only 60% recovered (1680/2812). In both cases, however, activity in the washed pellets was a small fraction of the unwashed outer-segment activity. Whether differences in recovery of cyclic AMP and cyclic GMP phosphodiesterase activities reflect different enzymes cannot be evaluated with the present data. The residual activity of cylic GMP phosphodiesterase activity in the washed pellet was unaffected by assaying it in hypotonic sucrose, as opposed to isotonic sucrose in the other assays.

If the concentrated supernatant fraction was added back to the washed pellet the light effect was not restored, indicating that a factor or factors may have been lost during the procedure. Neither light nor electron microscopy

revealed differences in morphology of the unwashed and washed outer-segment fragments before or after freezing.

Discussion

The association with isolated bovine photoreceptor outer segments of a light-stimulated, ATP-requiring cyclic GMP phosphodiesterase activity has been confirmed, as Bitensky's group [8] at Yale has reported for frog retinal outer segments. Light sensitivity was found in the cyclic GMP-hydrolyzing enzyme but not, to date, in the cyclic AMP enzyme. Lineweaver-Burk plots revealed two K_m values for cyclic AMP phosphodiesterase, but thus far only one for cyclic GMP phosphodiesterase.

In deviance from the Yale group's [8] report for cyclic AMP phosphodiesterase activity in frog outer segments, cyclic GMP phosphodiesterase activity in bovine outer-segment fragments was directly related to the amount of unbleached rhodopsin which was bleached experimentally. The degree of structural integrity may be important in determining enzyme activity, since sonication dramatically diminished both light sensitivity and dark activity, as did storage at −20° C. However, freezing at −76° C and the homogenization used during the outer-segment preparation did not affect either light or dark activity. These facts, taken together with the close association of cyclic nucleotide phosphodiesterases with rhodopsin following gradient centrifugation (refs 14 and 17, and Zimmerman, W.F., personal communication) might be interpreted as evidence that cyclic nucleotide phosphodiesterase is functionally associated with the rod outer segments in vivo.

The removal of phosphodiesterase activity by washing raises the question of the native location of the light-activated enzyme. One interpretation is that the enzyme is located on the outside of the plasmalemma, though its function there would be indeterminate. If it is located there, it may also have been adventitiously bound during isolation, and not be native to that location. Another possibility is that sucrose might wash the enzyme out of the interior of the outer-segment fragments. Though sucrose may permeate the plasmalemma, it does not permeate the discs [18]. The washing experiments, therefore, appear to exclude the possibility of an intradiscal location, though perhaps not of some other location inside the plasmalemma.

Regardless of location, the fact that the enzyme can be quantitatively recovered after removal from the outer-segment fragments suggests the feasibility of eventual resolution of the relationship between the enzyme and rhodopsin. This task should be significantly easier because of the availability of large preparations derived from cattle retinas as compared with very limited preparations from frog retinas.

Acknowledgements

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